GRADIENT ELUTION, IMPROVED SEPARATIONS AND ANALYTICAL FIGURES OF MERIT IN HIGH PERFORMANCE LIQUID CHROMATOGRAPHY USING ELECTROCHEMICAL DETECTION

BY

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To my parents, brother and sisters

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KEY TO ABBREVIATIONS

BPC	Bonded-phase chromatography
CMC	Critical micelle concentration
CV	Cyclic voltammetry
DC	Direct current
DP	Differential pulse
EC	Electrochemical detection or electrochemical detector
GC	Gas chromatography
GCE	Glassy carbon electrode
GE	Gradient elution
HDV	Hydrodynamic voltammogram
HPLC	High performance liquid chromatography
IP	Ion-pair
LC	Liquid chromatography
LC/EC	Liquid chromatography with electrochemical detection
LC/MS	Liquid chromatography mass spectrometry
LDR	Linear dynamic range
LLC	Liquid-liquid chromatography
LOD	Limit of detection
MSRTP	Micelle-stabilized room-temperature phosphorescence
NMR	Nuclear magnetic resonance
NP	Normal pulse
NRDP	Non-ramping differential pulse

PAH	Polyaromatic hydrocarbon
PAR	Princeton applied research
RP	Reversed-phase or reverse-phase
RP-HPLC	Reversed-phase high performance liquid chromatography
RP-LC	Reversed-phase liquid chromatography
RP-LC/EC	Reversed-phase liquid chromatography with electrochemical detection
SDS	Sodium dodecyl sulfate
WE	Working electrode

Abstract of Dissertation Presented to the Graduate Council of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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The goal of this work was an improvement and better understanding of electroanalytical methods used as liquid chromatographic detection techniques. Particularly, methods which would allow the use of chromatographic gradient elution with electrochemical detectors were investigated. Both amperometric and pulse techniques were investigated with traditional hydroorganic mobile phases. The use of gradient elution with micellar mobile phases was shown to allow compatibility with electrochemical detectors. A further comparison of analytical figures of merit was made between hydroorganic and micellar mobile phases using electrochemical detection

A comparison of baseline shift during gradient elution with amperometric and nonramping differential pulse (NRDP)

methods was performed using both glassy carbon and gold electrodes. The composition of the mobile phase is virtually constant during one pulse with gradient elution, but high residual current changes preclude routine use of this technique. A more stable baseline was achieved with an amperometric detector and a glassy carbon electrode, than it was otherwise with NRDP method. A higher concentration of phosphoric acid in the organic modifier as opposed to equal concentrations in both modifier and water produced a more stable baseline during gradient elution using a glassy carbon electrode and an amperometric detector.

The hydrodynamic voltammograms and analytical figures of merit for phenol, two B-6 vitamins, and polyaromatic hydrocarbons were compared in micellar and hydroorganic mobile phases. The limit of detection in both mobile phases was comparable, whereas the upper limit of linear dynamic range was greater in micellar mobile phases.

Gradient chromatograms for separation of phenolic compounds and B-6 vitamins with an anionic surfactant and phenolic compounds with a nonionic surfactant using amperometric detection are shown. Selectivity of micellar mobile phases toward B-6 vitamins changes with surfactant concentrations.

A rapid separation of thyroxine and related thyroid hormones is shown using gradient elution and electrochemical detection. A five minute isocratic separation of thyroxine and three related hormones is also reported. Limits of

detection are in the sub-nanogram range with an upper limit of linear dynamic range of 500 to 1000 nanograms for these compounds. Analysis of levothyroxine sodium tablets and injectable intravenous samples is described.

CHAPTER ONE

INTRODUCTION

Chromatographic methods can be classified according to a number of schemes. The major one is based on the state of the mobile phase. If the mobile phase is a gas, the method is called gas chromatography (GC). If the mobile phase is a liquid, the method is named liquid chromatography (LC). Liquid and gas chromatography are each divided according to the nature of the stationary phase. When the solid stationary phase has adsorption properties, the process is called adsorption chromatography, and when the stationary phase is a liquid supported by an inert matrix, the process is called partition chromatography.

Special classifications have also been introduced. For example, chromatography can be classified according to whether the stationary phase is present as a thin-layer, a paper, or a column. Chromatography can alternatively be classified with respect to the flow of the mobile phase; this classification includes one-way, two-dimensional, and radial chromatography. According to the mechanism of the retention, i.e., the interaction between solutes and the stationary phase, chromatographic methods are classified as adsorption, partition, ion-exchange, and gel permeation.

Finally, the chromatographic technique may be classified according to the kind of sample introduction onto the stationary phase and migration through the system. This gives rise to development, elution, displacement and frontal analysis chromatography. The first two techniques are the most common, while the last two methods are relatively specialized and, therefore, of limited value and use.

In 1941, Martin and Synge [1] were led to contrive a scheme of liquid-liquid chromatography for separation of the amino acids of a wool hydrolyzate with a countercurrent extractor [2]. To improve the efficiency of the countercurrent method, Martin and Synge considered a means of immobilizing one phase while the second phase flowed over it in such a manner as to maximize the contact of the immiscible liquids at the interface. Martin believed this would facilitate the rapid distribution, or partitioning, of the solutes between the two phases. The efficiency of their partitioning column was almost 10^4 times greater than that of the countercurrent method [3].

The difference between modern liquid chromatography and traditional column chromatography (whether adsorption, partition or ion-exchange) involves improvements in equipment, materials, technique, and the application of theory. Modern liquid chromatography provides more convenience, better accuracy, higher speed, and the ability to carry out difficult separations. At the beginning of the 1970's, the modern form of liquid chromatography was named high pressure

liquid chromatography (HPLC). Later, the P for "pressure" was replaced by P for "performance." The reason was the appearance of microparticles that allowed researchers to perform, at a lower pressure drop, the same efficient and rapid analyses done with other supports.

In conventional liquid-liquid chromatography (LLC), the stationary phase is a bulk liquid, mechanically held to the support by adsorption. In recent years, organic phases are chemically bonded to the support, leading to a separate LC method called bonded-phase chromatography (BPC). Bonded-phase chromatography is the most widely used in modern LC, and many laboratories use BPC columns for their LC separation. In contrast to LLC, bonded-phase chromatography packings are quite stable because the stationary phases are chemically bound to the support and cannot be easily removed or lost during use. The availability of a wide variety of functional groups in BPC packings allows for both normal- and reversed-phase chromatography.

Polar BPC packings are used for normal-phase separations. Samples of moderate to strong polarity are usually well separated by normal phase-chromatography. The mobile phase in normal phase chromatography is a hydrocarbon solvent such as hexane, heptane, or isooctane, plus small amounts of a more polar solvent. The mobile phase strength can be varied for a given application by varying the concentration of the more polar solvent component. Reverse phase

BPC normally involves a relatively nonpolar stationary phase (e.g., C_8 or C_{18} hydrocarbon) used in conjunction with polar (e.g., aqueous) mobile phases to separate a wide variety of less polar solutes.

The general reaction for preparing bonded-phase packings on silica-based supports to produce siloxanes is:

$$\text{Si-OH} + \begin{matrix} \text{ClSi(Me)}_2\text{R} \\ \text{or} \\ \text{R'OSi(Me)}_2\text{R} \end{matrix} \longrightarrow \text{Si-O-Si(Me)}_2\text{R} + \text{HCl}$$

where R is the desired organic moiety and, for reversed-phase (RP) packings, is an n-alkyl chain (n = 2,8,18). This reaction is based on silanol groups on the surface of the siliceous support. Fully hydrolyzed silica contains about 8 μmol of silanol groups per m^2. Because of steric hindrance, a maximum of about 4.5 μmol of silanol groups per m^2 can be reacted at best [4], and an end-capping process usually can be done to cover most of the residual silanols by a reaction similar to the one above using chlorotrimethylsilane. The concentration of the organic moiety per m^2 of BPC packings depends upon the surface area of the packing particles (e.g., pellicular or porous support). Siloxane bonded-phase packings are available with pellicular or totally porous supports. Bonded-phases of this type are hydrolytically stable throughout the pH range 2-8.5. Due to the different methods for the preparation of BPC packings and shielding the residual silanol groups by end-capping, the surface coverage and overall volume of the organic

stationary phase tends to show large differences from manufacturer to manufacturer, and even from lot to lot. Without end-capping of the residual silanol groups, a mixed-retention mechanism can result and lead to asymmetric peaks.

Increasing the alkyl chain length on BPC results in an increase in selectivity and retention times [5,6] (when the columns were compared) using the same mobile phase. The study of chain length (C_8-C_{22}) of bonded organic phases showed that the selectivity [7] depended on the chain length of the bonded phase and on the molecular structure of the solute. The same study showed that the utilization of long chain phases made it possible to reduce the water content of the water:methanol mobile phase, which increases the efficiency and loading capacity. Hemetsberger et al. [8,9] studied the behavior and the effect of structure of bonded phases. Kikta and Grushka [10] studied the retention behavior on alkyl bonded phases as a function of chain length, surface coverage, solute type, mobile phase composition, and temperature. Colin and Guiochon [11] compared the resolution of RPC packings (C_6-C_{22}). As a result, the shorter length alkyl chain columns generally gave the worst resolution and efficiency. A comparative study on the separation, efficiency under optimum mobile phase conditions with three different mobile phases, and three groups of solutes on three commercially available alkyl bonded phases (C_2, C_8 and C_{18}) was done by Haleem [12].

In high-performance liquid chromatography (HPLC), as in all analytical methods, the trend is to do it faster and

cheaper, make it more selective and sensitive, and combine it with other methods. Selection of column type in LC has been more restricted because of the viscosities and solute diffusivities in the liquid phase which are orders of magnitude different from the values in GC. The most efficient columns presently used in LC are those packed with totally porous small particles (with particle size down to a few micrometers). Although substantial reductions of the plate height are achieved while decreasing the particle size, there are some practical limits to this procedure. As pointed out by Halasz [13], there are difficulties in the uniform packing of very small particles, as well as the problem with evolved heat of friction.

Microbore HPLC as now commercially available uses 1- to 2-mm diameter columns. The next generation of columns, studied only in research laboratories for the past few years, may be inner-coated microtubular or packed microcapillary columns, just 50 μm in diameter. The advantage of these would be hundreds of thousands of theoretical plates in a very long length at low cost. Eluents from these columns also could be fed into a mass spectrometer with low interference from the solvent. These small diameter columns often require new instruments or modifications of existing instruments to meet new needs in injection, pumping, or detection.

The rapid progress in HPLC places great demands on detection techniques. Unfortunately, the highly developed

GC detectors are mostly useless at this time in LC because of principal differences between LC and GC. An ideal detector should be universal; however, the great diversity of systems to be analyzed makes the construction of a sensitive universal detector impossible, and thus detectors monitoring various physicochemical properties of substances are employed, the optimal detection conditions being determined specifically for each system. It is possible to measure either bulk properties, which depend on the variation of the composition of the system (e.g., refractive index, electrical conductance, etc.), or properties that selectively characterize certain components in the mobile phase (such as the absorbance at a certain wavelength, fluorescence, electric current at a certain electrode potential, etc.). The bulk property detectors are universal detectors, and they require that the properties of the solutes be substantially different from those of the mobile phase to attain sufficient signal changes during detection. The bulk property detectors are usually less sensitive and subject to a higher noise than the measurement of specific properties of the solutes, and they are rarely compatible with gradient elution. On the other hand, the measurement of specific properties requires that the mobile phase yields the lowest possible signal under the given conditions.

Currently available commercial LC detectors are based on a number of detection principles, including absorbance, fluorescence, refractive index, electrochemical reaction,

and mass spectrometry. A number of these detectors are likely to be improved in the next few years. For instance, new types of LC/MS interfaces will probably appear, and currently available interfaces should be further refined. Nuclear magnetic Resonance (NMR) detectors should materialize, and GC detectors are being seriously considered for their applicability to LC detection. The development of laser based detectors [14] for chromatography is in progress and in many cases offer better sensitivity and selectivity than conventional LC detectors. The complexity and expenses of the laser based detectors have delayed the acceptance of these detectors in the laboratory.

Attempts to use electrochemical detection of molecule(s) in effluents from chromatographic columns were made long before the advent of HPLC. The first papers dealing with polarographic detectors were those of Drake [15] and Kemula [16]. Present electrochemistry offers a large group of methods that can be used for continuous detection of substances [17]. The field of electrochemical HPLC has been reviewed several times [17-19]. A survey of scientific papers on LC detector usage during the 1980-81 period showed that 4.3% of LC analyses were based on electrochemical detectors [20].

The suitability of electrochemical detection to a given problem ultimately depends on voltammetric characteristics of the component(s) of interest in a suitable mobile phase and a suitable working electrode surface. Electrochemical

detection is more limited with respect to the mobile phase composition than other LC detection methods because of the fact that a complex surface reaction which depends on the medium is involved.

Direct electrochemical detection is not likely to be useful in normal-phase chromatography since nonpolar organic mobile phases are not well suited to many electrochemical reactions. The HPLC stationary phases of choice clearly include all ion-exchange and reverse-phase (RP) materials since these are compatible with polar mobile phases containing some dissolved ions. The ionic strength, pH, electrochemical reactivity of the mobile phase and background electrolyte, and presence of electroactive impurities (dissolved oxygen, halides, trace metal ions) are all important considerations.

The choice of electrode material is one of the important considerations, because of the ruggedness, potential range, residual current, and long-term stability requirements. Electrode materials such as platinum, glassy carbon, gold, and mercury films may work well in some cases but may be disastrous in others. These electrodes are subject to complicated surface renewal problems but are not mechanically awkward devices such as the dropping mercury electrode. With all limitations mentioned, liquid chromatography with electrochemical detection (LC/EC) has three distinct advantages for applicable systems, namely, selectivity, sensitivity and economy.

CHAPTER TWO

THEORY AND BACKGROUND

Basic Parameters in Chromatography

Capacity Factor (k')

The capacity factor is equal to n_s/n_m, where n_s and n_m are the number of moles of solute in the stationary and mobile phase, respectively. Therefore, k' can be written according to the following:

$$k' = \frac{n_s}{n_m} = \frac{[X]_s V_s}{[X]_m V_m} = \frac{KV_s}{V_m} = \frac{t_R - t_0}{t_0} \qquad (1)$$

where

$[X]_s$ = concentration of solute X in the stationary phase

$[X]_m$ = concentration of solute X in the mobile phase

V_s = volume of the stationary phase

V_m = the total volume of the mobile phase within the column

K = distribution constant

t_R = retention time of solute X

t_0 = time for mobile phase or other unretained molecules to pass through column

Peak Resolution (R_s)

By convention, peak resolution, R_s, is defined as the ratio of the distance between the two peak maxima (Δt) to the mean value of the peak width at base (W_b).

$$R_s = \frac{\Delta t}{\frac{W_{b1} + W_{b2}}{2}} = \frac{2\Delta t}{W_{b1} + W_{b2}} \qquad (2)$$

For two closely spaced peaks, one can assume that the two peak widths are the same, and W_{b2} can be used instead of the mean.

Efficiency (E)

Efficiency of chromatography, E, is defined as peak retention time divided by peak width at base [21].

$$E = \frac{t_R}{W_b} \qquad (3)$$

The theoretical plate number, N, contains the same information as E.

$$N = \left(\frac{t_R}{\sigma}\right)^2 = 16\left(\frac{t_R}{W_b}\right)^2 = 16E^2 \qquad (4)$$

or

$$N = 5.54\left(\frac{t_R}{W_h}\right)^2 \qquad (5)$$

where σ and W_h are the standard deviation and peak width at half height of the peak, respectively. An equation derived

by Foley and Dorsey [22] can be used for the calculation of the number of theoretical plates of skewed peaks in a chromatographic system (N_{sys}).

$$N_{sys} = \frac{41.7\left(t_R/W_{0.1}\right)^2}{B/A + 1.25} \qquad (6)$$

where t_R, $W_{0.1}$ and B/A are retention time, peak width at 10% of peak height, and asymmetry factor, respectively.

Control of Separation in Liquid Chromatography

The key to separating components of a mixture is to control resolution.

$$R_s = \tfrac{1}{4}(\alpha-1)\sqrt{N}\left[\frac{k'_2}{(1+k'_2)}\right] \qquad (7)$$

An increase of separation factor, α, which is the ratio of two capacity factors, k'_2/k'_1, results in a displacement of one band center relative to the other and a rapid increase in R_s. Increasing the number of theoretical plates narrows the bands while increasing the peak height. For early eluting peaks, an increase in k' can provide a significant increase in resolution, however, with increasing k', band height decreases and separation time increases.

The available options for increasing, α, in order of decreasing utility are: change of stationary phase, change of temperature, and special chemical effects. Increasing the number of theoretical plates can be done by increasing column length and decreasing flow rate for a given column.

Capacity factor can be increased by increasing the volume of stationary phase, decreasing the strength of mobile phase, and decreasing temperature.

Gradient Elution (GE)

The most convenient way to separate a complex mixture of solutes is to use gradient elution. Gradient elution in LC is similar to temperature programming in GC, except that the composition of the mobile phase is changing during separation time. To do GE, one needs at least two different solvents (binary gradient). One of the solvents has higher eluent strength, and usually its percentage increases during the gradient. Ternary gradients using three solvents are sometimes used in LC. Multisolvent gradients are rarely required in LC, and because of the complexity, one usually avoids the use of multisolvent gradients.

The purpose of gradient elution is to resolve early eluting bands and decrease the retention time of strongly retained compounds in comparison to isocratic elution. To achieve this, the gradient must start with a weak mobile phase, and the strength of the mobile phase increases during the chromatographic run. Because of decreasing retention times for late eluting bands, these peaks are greatly sharpened in gradient elution when compared to isocratic elution, and sensitivity for these bands is therefore much improved. Gradient elution increases the peak capacity for a mixture that contains a large number of individual

components and improves the peak shapes for bands that would tail in isocratic elution. The gradient shape for binary solvents can be linear, concave, convex, or any other shape. The appropriate gradient shape is dependent on LC methods, sample, and solvent composition. The steepness of the gradient is the mobile phase strength change with time.

Limit of Detection

The limit of detection represents the ability of an analytical method for quantification of a chemical component in terms of concentration or absolute amount. In most analytical methods, the limit of detection, C_L, is defined according to the following equation.

$$C_L = \frac{KS_b}{m} \tag{8}$$

where S_b and m are the standard deviation of the blank and slope of the calibration curve, respectively. On a statistical basis, K = 3 is the most appropriate number for calculation of the detection limit. For more information about detection limits, one is referred to articles by Kaiser [23] and Winefordner [24].

To calculate the detection limit in LC, the peak to peak noise is measured while mobile phase passes through the column. In a normal distribution, peak to peak or random noise in LC is considered to be 5 times the standard deviation of the blank, as in equation (8). Detection limit in

LC is usually defined as 3 times the peak noise or 3/5 of the peak to peak noise divided by the slope of the calibration curve. The latter was used for reporting detection limits in this dissertation.

Principles of Electrochemical Detection

The most common detector in LC/EC is the amperometric detector which measures the current at constant potential. The amperometric detector is more sensitive and less complex than the coulometric detector. In a coulometric detector, the amount of electricity for complete electrochemical conversion of the analyte is measured at constant potential. The lower sensitivity of the coulometric detector is due to geometrical requirements necessary for complete electrochemical conversion in a flowing stream. The requirement of larger surface area of working electrode causes higher background current and noise which reduces the signal to noise ratio in comparison to amperometric detector.

The technique of electrochemical detection is based on electroactivity of components eluted from the column. Sometimes it is possible to detect nonelectroactive components by pre- or post-column derivitization. Selecting the applied working electrode potential is the primary requirement in amperometric detection. The applied potential should be held at the minimum value at which the current reaches the limiting-current plateau of the analyte ($E_{plateau}$), however, in most of the cases, $E_{plateau}$ is

different for different components of a mixture to be analyzed. In this case the analyst should choose the optimum applied potential to analyze all components of interest in the mixture. The applied or analytical potential for an analyte can be determined by a hydrodynamic voltammogram (HDV). In an HDV, current is measured versus applied potential for analyte injected into an LC amperometric detection system. The $E_{plateau}$ can be precisely identified by HDV measurement under analytically useful LC conditions, but it is a time-consuming process, due to the time required for the baseline to stabilize after each change of electrode potential. The time required for stabilization of the baseline is dependent upon the mobile phase composition and flow rate. In the case of a glassy carbon electrode, for a change of 0.1 V in applied potential, it takes 15 to 30 min to get a stable baseline. Cyclic voltammetry (CV) is a much faster method for determining $E_{plateau}$, which usually has a higher magnitude than the CV peak potential (E_p) under typical measurement conditions for slow electron transfer reaction. Data from CV and $E_{plateau}$ can be related via a simple empirical equation [25].

Ion-pair Chromatography

The extraction of ionized solutes into organic phases has been well known for a number of decades. To extract ionized species from aqueous solution, an ion-pairing reagent of opposite electrical charge is added to the aqueous phase

resulting in ion-pairing between the solute ion and pairing ion. The resultant complex which has a net low electrical charge or polarity can easily be extracted by an organic phase. To separate ionic species by reversed-phase HPLC, an ion-pairing reagent can be added to the mobile phase. Ion-pairing reagents can also be used as a probe to detect and quantify compounds which cannot be directly detected [26,27]. The following scheme shows overall phase transfer of ion pairs.

$$\underset{\text{aqueous phase}}{A^+ + B^-} \rightleftharpoons \underset{\text{organic phase}}{(A,B)}$$

In the case of reversed-phase chromatography, the organic and aqueous phases on the above scheme are considered to be stationary and mobile phases, respectively. The most popular ion-pair reagents for cationic solutes are long-chain alkyl sulfonate ions which are usually added to the mobile phase to enhance separation of oppositely-charged sample ions.

The exact ion-pairing mechanism for the separation of ionic samples is still uncertain. Three popular hypotheses are: (1) the ion-pair model, (2) the dynamic ion-exchange model, and (3) the ion-interaction model. The ion-pairing model stipulates that the formation of an ion-pair occurs in the aqueous mobile phase which is in agreement with solvophobic theory [28], while the dynamic ion-exchange model states that unpaired lipophilic alkyl ions adsorb onto the nonpolar stationary phase, causing the column to behave as

an ion exchanger [28]. The ion-interaction model is based upon conductance measurements. It proposes that neither the ion-pairing nor the ion-exchange model can explain the experimental data in a consistent way [28]. The ion-interaction model assumes that a primary layer of lipophilic ion covers the surface of the stationary phase which is in dynamic equilibrium with the bulk eluent. In the vicinity of this primary layer exists a secondary layer of opposite charge creating an electrical double layer on the surface. The retention of the ionic components is due to the electrostatic force between these ions and the primary layer, as well as an additional (sorption) effect onto the nonpolar stationary phase.

Micellar Chromatography

It is well known that surfactants, detergents, or surface active agents are amphiphilic molecules (i.e., molecules in which a hydrophobic tail is joined to a hydrophilic head-group). Surfactants can be anionic, cationic, nonionic, and zwitterionic. Above a certain concentration, surfactant molecules associate in aqueous solution to form large molecular aggregates of colloidal dimensions termed micelles. The concentration threshold at which a surfactant starts to form micelles is called the critical micelle concentration (CMC), and the number of surfactant molecules in a micelle is called the aggregation number. The aggregation number and the CMC differ from one surfactant to

another, and even for the same surfactant in different media. At concentrations greater than the CMC, a dynamic equilibrium exists between the surfactant molecules and micelles. The general size and shape of the particular micelle depend on the aggregation number.

The term normal micelles is used for surfactant aggregation in aqueous media. The hydrophilic head groups are directed toward and in contact with aqueous solution to form a polar surface, while the hydrophobic tails are directed away from the water to form a central nonpolar core. In nonpolar solvents, the surfactant aggregates are termed reversed or inverted micelles. In these micelles, polar head groups are concentrated in the interior of the aggregates and hence form a central hydrophilic core, while the hydrophobic tail moieties extend into and are in contact with the bulk nonpolar solvent.

The solubilizing power of micellar systems is one of its most important aspects. This refers to the ability of micelles to solubilize a wide variety of solutes that are insoluble or only very slightly soluble in the bulk solvent alone. The solubilization of solutes in micellar systems is a dynamic process and depends upon such factors as the temperature, the nature of solutes, the surfactant concentration, and the type of micelles. The amount of solute solubilized is usually directly proportional to the concentration of micelles. The solubilization of a solute at a micelle site is dependent upon the type of solute and the

nature of the micelle. In a normal micelle, a nonpolar solute is thought to be located near the center of the hydrophobic core, while an ionic solute is adsorbed on the polar micellar surface.

According to the properties of micellar systems mentioned above, micellar solutions can be used as mobile phases in HPLC. The normal micellar solution can be used as the mobile phase in reversed-phase HPLC, while reversed micellar solutions are compatible with normal-phase HPLC. Equations for partitioning behavior of solutes with micellar mobile phases in LC have been derived by Armstrong and Nome [29]. From these equations, one can calculate the partition coefficients of solutes between water and micelles, between the stationary phase and water, and between micelles and the stationary phase. One of the drawbacks of micellar mobile phases in RP-HPLC is its poor efficiency in comparison to hydroorganic mobile phase, however, Dorsey et al. [30] showed that by the addition of 3% of propanol and a temperature of about 40°C, micellar mobile phases can approach efficiencies of hydroorganic mobile phases. Possible advantages of micellar mobile phases over hydroorganic mobile phase are: (1) the unique selectivity of micellar mobile phases toward different types of solutes, (2) the economy when compared to hydroorganic mobile phases, and (3) the simplicity of purification of the crystalline surfactants compared to organic solvents.

CHAPTER THREE

EXPERIMENTAL

Cyclic Voltammetry Systems

To find an approximate analytical potential for solutes of interest, a CV-1A cyclic voltammetry instrument and an electrochemical cell made by Bio Analytical Systems, Inc. (West Lafayette, Indiana) were used. The working and reference electrodes were glassy carbon and Ag/AgCl, respectively. Before running the CV experiments, the sample solutions were purged for 20 min with helium. Cyclic voltammetry was then carried out in an inert helium atmosphere. A Plotamatic MFE-715 (MFE, Salem, New Hampshire) X-Y recorder and digital voltmeter were used to record the cyclic voltammograms.

Liquid Chromatography System

The solvent delivery unit used during the chromatographic run was a Waters 6000 A (Waters Associates, Milford, Massachusetts), an Altex model 322 gradient liquid chromatograph with two model 100 A pumps (Altex Scientific, Berkeley, California), or a Spectra-Physics SP 8700 solvent delivery system (Spectra-Physics, Santa Clara, California).

The injection valve was either an Altex 210 or Rheodyne 7125 (Rheodyne, Cotati, California) with 5, 10 and 20 µL loops. Various columns--an Altex Ultrasphere octyl, 250 x 4.6 mm; an Altex Ultrasphere ODS, 150 x 4.6 mm; and Rainin Microsorb octyl column, 150 x 4.6 mm--were employed.

Electrochemical Detector

The electronic controller was an LC-4 amperometric controller from Bio Analytical Systems, Inc. or Princeton Applied Research (PAR, Princeton, New Jersey) model 174 polarographic analyzer. The electrochemical cell used was either from Bio Analytical Systems, Inc., with glassy carbon as working electrode, or a porous membrane separator with gold as the working electrode, a generous gift of K. A. Rubinson. The latter working electrode was used only in work of Chapter Four of this dissertation to compare electrode material, while the former was used from Chapter Four through Chapter Six. The reference electrode for both cells was Ag/AgCl from Bio Analytical Systems, Inc.

The electrochemical cell from Bio Analytical Systems, Inc. [31], is a thin-layer cell, as shown in Figure 1. The thin-layer cell, reference electrode compartment, clamp, and waste and connecting tubes are preassembled as one unit. Addition of a reference electrode to this unit completes the detector cell. All of the detector cell components have been machined to accept standard plastic tube end

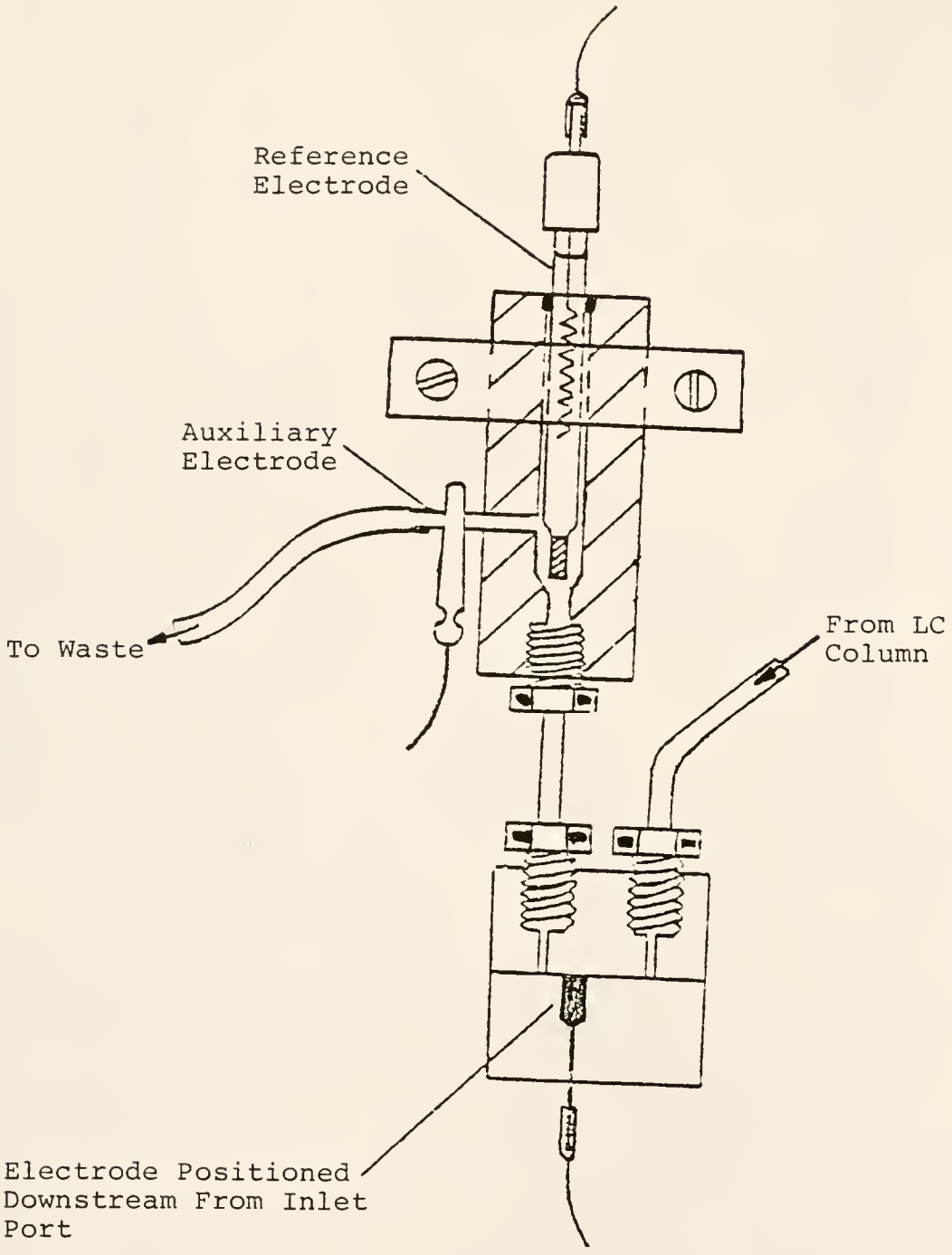


Figure 1. Electrochemical Cell with Glassy Carbon Electrode

fittings (¼-28 thread) as supplied by BAS, Altex, LDC, Omnifit, and others; therefore, the detector cell is directly compatible with commercial HPLC systems.

The porous membrane separator contained gold as the working electrode [32]. The 0.5 mm gold wire of 5 cm length was covered with porous polymer tubing which can be attached to the outlet of the column in an LC system. The gold electrode covered with porous membrane was cemented into a small reservoir which contained an external electrolyte solution (1 or 2 M KCl), auxiliary (Pt), and reference electrodes. To prevent any damage to the porous membrane gold electrode, it was soaked in 1 M KCl for several hours before running mobile phase through it from an LC system.

Pretreatment of Glassy Carbon Electrode

It is well known that the sensitivity of glassy carbon electrodes decreases with use. Decreasing activity at the glassy carbon electrode surface can be due to adsorption of analytes, mobile phase components, and byproducts of redox reactions onto the electrode surface as well as formation of electroactive species, such as carbonyl and hydroxy groups, from components in the electrode material. The adsorption of these components at the electrode surface may form a polymeric film which would decrease electrode response; however, whatever the source of deactivation might be, it is necessary to clean and reactivate the electrode surface by mechanical, chemical, electrochemical, or by a

combination of these methods. More details on pretreatment and the study of the glassy carbon electrode surface are given elsewhere [33-35].

A deactivated electrode surface produces a large residual current as well as a noisy baseline which affects the accuracy of analytical application and increases the detection limits. For reactivation or pretreatment of a glassy carbon electrode surface the following procedure has given satisfactory results. This procedure would be used if analytical potential is positive or anodic.

(1) The cell was dismounted and electrode surface was washed with methanol.

(2) A few drops of a slurry of alumina [0.1 μm, Gamal, Grade B, Fisher Scientific Company (Fairlawn, New Jersey)] in water were placed at the surface of the electrode. The surface was then carefully polished with Buehler LTD polishing paper (Evanston, Illinois) for 2-3 min.

(3) The electrode surface was rinsed with methanol and polished with the same polishing paper which was soaked in methanol, and then the cell was assembled.

(4) An anodic potential (0.1-0.2 V higher than that of analytical potential) was applied for 3-4 min while mobile phase passed through the electrochemical cell.

(5) A cathodic potential (-0.8 V vs. Ag/AgCl) was applied for 2-3 min while mobile phase passed through the electrochemical cell.

(6) The steps of (4) and (5) were repeated, and then the potential was set at the analytical potential. The potential switch was turned off before changing from the anodic-applied potential to the cathodic-applied potential or vice versa.

Reagents

Methanol and acetonitrile, HPLC grade (Fisher Scientific Company, Fairlawn, New Jersey) were employed as organic mobile phase components. Methanol, HPLC grade, was used as the solvent for test solutes. Deionized water, another mobile phase component, was purified by a Barnstead Nanopure system (Sybron Corp.) The purified water then was irradiated for 24 hr by ultraviolet light in a model 816 HPLC reservoir (Photronix Corp., Medway, Massachusetts). The other chemicals comprising the mobile phases were: (1) reagent grade phosphoric acid (Scientific Products, McGaw Park, Illinois), (2) sodium perchlorate and Brij-35 (Aldrich Chemical Co., Milwaukee, Wisconsin), and (3) sodium dodecyl sulfate (SDS; Fisher Scientific Co.). The reagents mentioned above mostly were used as a component of the mobile phase or dissolved in the mobile phase. Other specific chemicals (e.g., sample solutes) will be mentioned in chapters related to them.

CHAPTER FOUR

SIGNAL CHANGE AND BASELINE SHIFT USING ELECTROCHEMICAL METHODS

In this chapter the following will be considered: (1) change of residual current with ordinary DC voltammetry, normal pulse (NP) voltammetry, and differential pulse (DP) voltammetry in isocratic elution HPLC; (2) effect of the background electrolyte and gradient elution on baseline shift using amperometric detection; and (3) effect of the electrode material on baseline shift using amperometric and non-ramping differential pulse (NRDP) voltammetry methods.

Pulse techniques such as DP and NP can be used as LC detection schemes if a constant pulse amplitude superimposes over an initial potential. This means that there is no potential ramp, and it is better to be called non-ramping pulse techniques. The term "pulse techniques" has been used instead of the term "non-ramping pulse techniques" in the literature, however. Fleet and Little [36] were among the first authors to be concerned with application of pulse techniques as an HPLC monitoring method using a mercury drop electrode (MDE). The idea was examined in practice by Swartzfager [37], using carbon paste as a working electrode. The advantages of pulse systems in monitoring have been discussed by Kissinger [19], who noted the

possibility of separating the monitored electrochemical system from systems with more positive or negative half-wave potentials and prolonging electrode service life by electrochemical cleaning. Electrochemical cleaning during the pulse operation is the subject of a patent by Fleet [38]. A versatile voltammetric detector with double polarization pulse and semi-differential scanning for LC is reported by Stastny et al. [39]. Pulse techniques also offer the advantage of decreased dependence of the measured current on flow-rate in comparison to amperometric techniques [37], because the short potential pulse duration minimizes the development of the diffusion layer of the electrode and dependence of the thickness of this layer on flow-rate. With all the advantages of pulse techniques (more selectivity, increased electrode stability, and less current dependence on flow-rate) over amperometric techniques, these techniques are limited by poor sensitivity and complex instrumentation. The residual current at a solid electrode is reportedly high [17]. A study of the change in magnitude of the residual current with different electrochemical methods using isocratic elution and with amperometric method using gradient elution was performed and will be reviewed. The residual current change during gradient elution with amperometric detection is a baseline shift due to the change in the composition of the mobile phase.

The electrochemical equipment used was a model 174 polarographic analyzer, an LC-4 amperometric controller, and

an electrochemical cell either with a glassy carbon electrode (GCE) or a porous membrane gold (Au) electrode.

Change of Residual Current with DC, DP and NP Voltammetry in Isocratic Elution HPLC

In differential pulse (DP) voltammetry and normal pulse (NP) voltammetry, the current is measured during a time interval of the pulse when the ratio of faradaic current to charging current is a maximum. The electrochemical cell can be considered as an RC-circuit, and thus the charging current decays exponentially. The purpose of these experiments was a comparison of the residual current change versus applied electrode potential with different electrochemical methods using isocratic elution.

The residual current change and decay current on a glassy carbon electrode with different percentage of acetonitrile as an organic modifier in water are shown in Figures 2 through 5. The data from these figures are reported in Table 1. All mobile phases contained 0.04 M $NaClO_4$ as a background electrolyte. The potential was scanned from 0.55 V (E_i) to 1.30 V (E_f) versus Ag/AgCl reference electrode.

The residual current change, $\Delta I = I_f - I_i$, is due to the difference between the current at the final potential (E_f) or the current at the end of the ramp, I_f, and the current at the initial potential, I_i. The residual current change, ΔI, can be considered to be due to the residual current difference at two different potentials. Decay

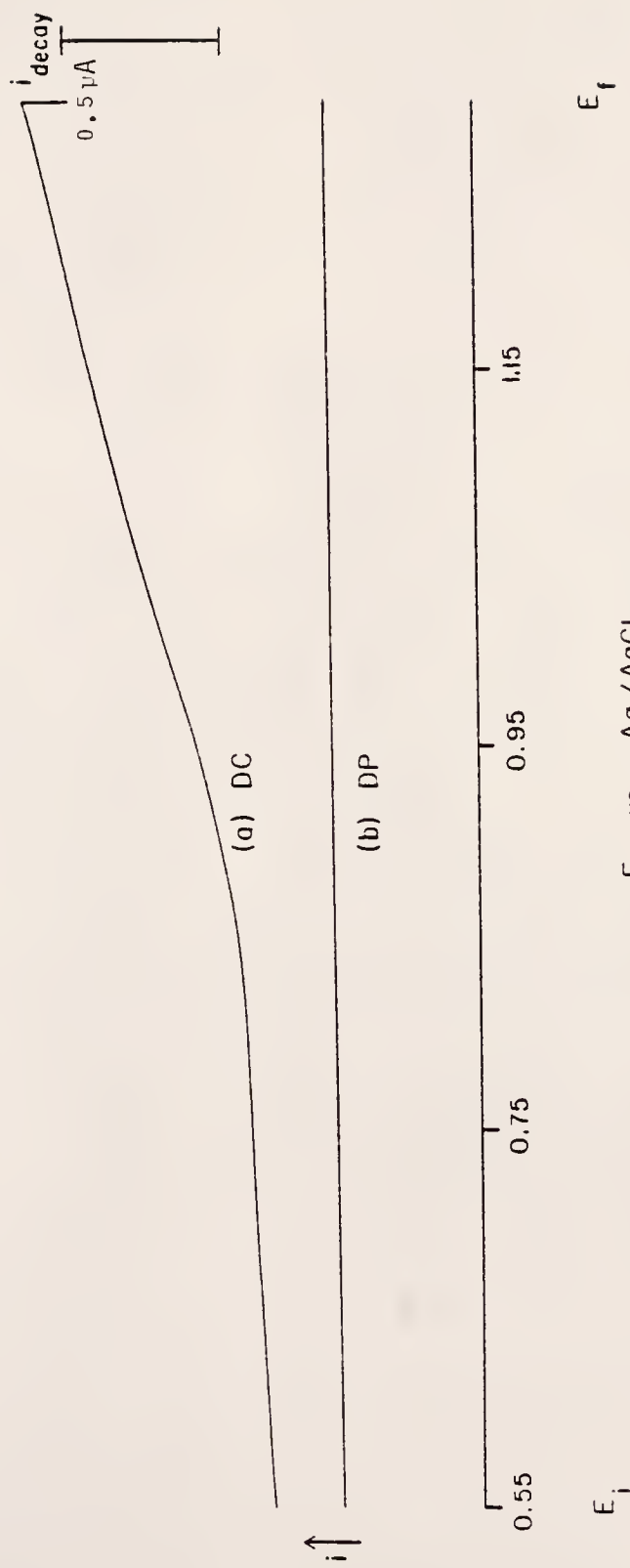


Figure 2. Residual Current Change and Decay Current with 5% CH_3CN

(a) DC voltammogram

(b) DP voltammogram

Other conditions are as in Table 1.

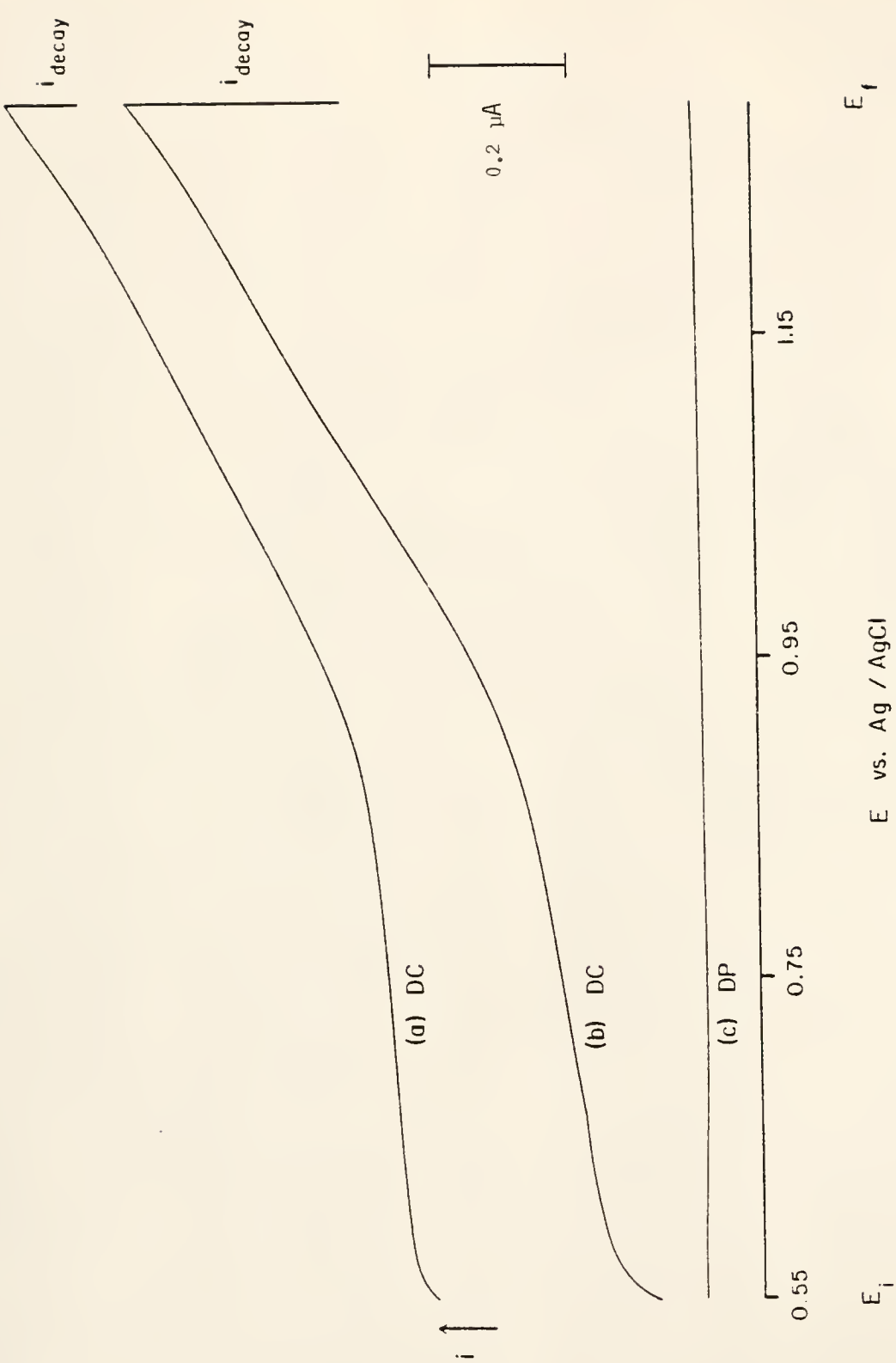


Figure 3. Residual Current Change and Decay Current with 50% CH_3CN

(a,b) DC voltammogram (c) DP voltammogram

Other conditions are as in Table 1.

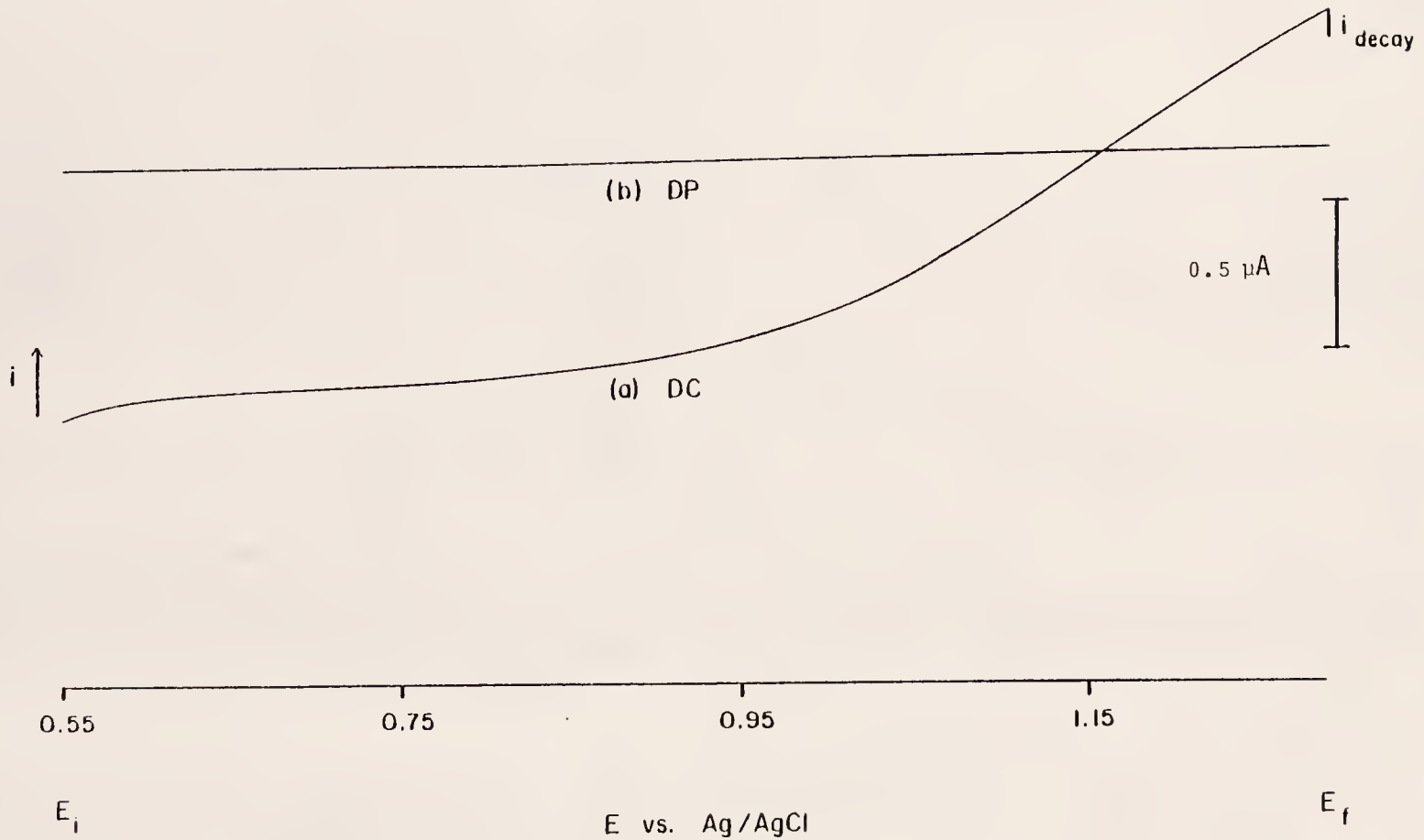


Figure 4. Residual Current Change and Decay Current with 95% CH_3CN

(a) DC voltammogram

(b) DP voltammogram

Other Conditions are as in Table 1.

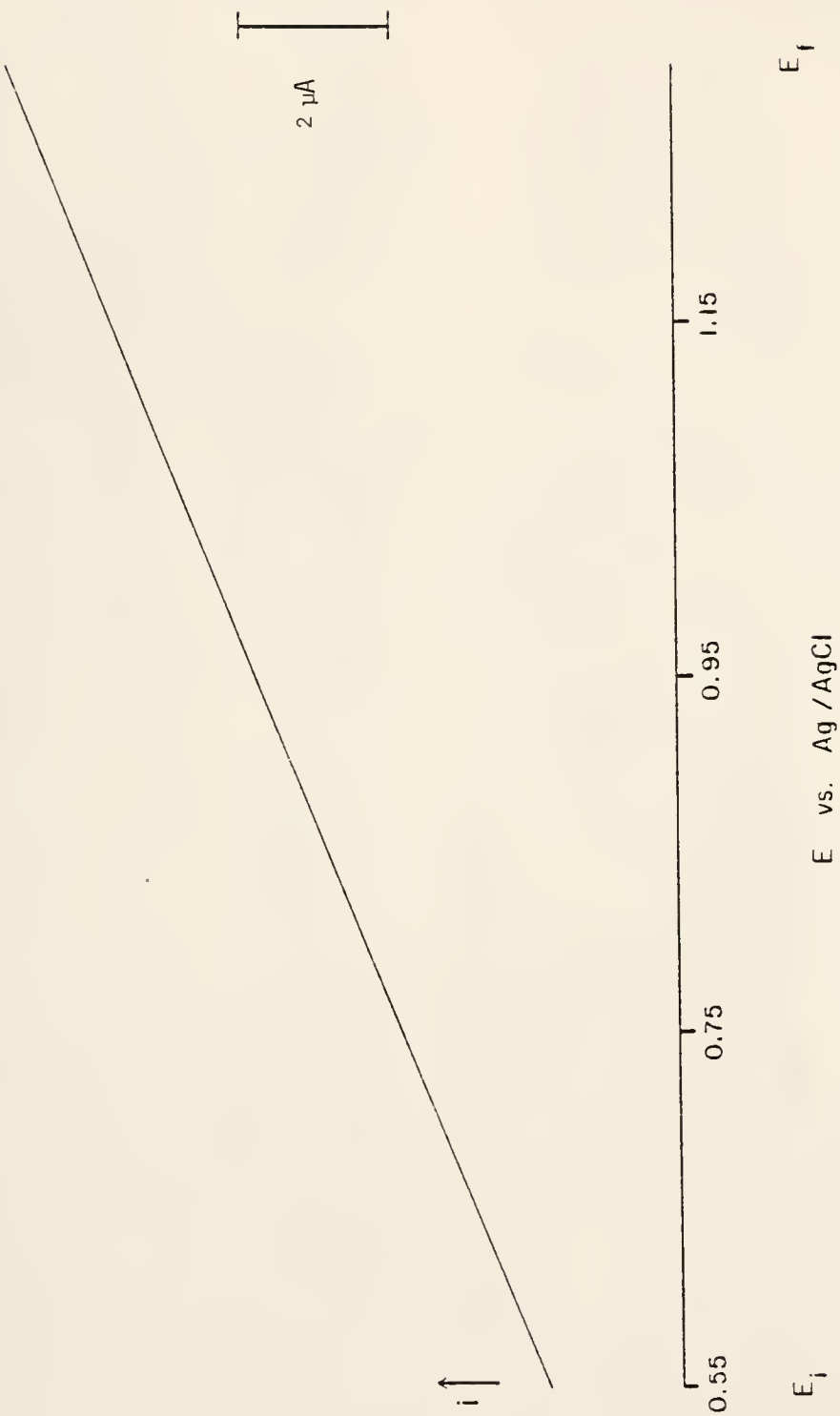


Figure 5. Residual Current Change by NP Voltammetry

Mobile phase: 95% CH_3CN

Other conditions are as in Table 1.

current is the amount of current depleted at the end of the ramp (E_f) within a short time. This current depletion might be considered as the charging current and decreasing current due to impurities. Since the charging current decays quickly (10^{-4} to 10^{-3} sec), it is impossible to find out the origin of decay current with a stripchart recorder.

The data in Table 1 show that on the DP mode the residual current change, ΔI, and decay current, i_{decay}, are in the limit of measurement error (measurement device was a ruler). This means that the currents sampled shortly before the end of the pulse and shortly before the rise of the pulse are almost the same, and no difference in charging current is sampled. On the DC mode, there is some current change during the scan (potential applied to the electrode changes with time) and some decay current at the end of the scan. Increasing the scan rate will increase both ΔI, and i_{decay} in the DC mode because the magnitude of charging current increases as the scan rate increases. In the NP mode, ΔI is much larger than that of the DC mode, while the decay current is in the limit of measurement error. The current varies linearly with the applied potential on the working electrode (WE) in the NP mode. The larger magnitude of ΔI in the NP mode as compared to the DC mode might be due to the surface clean up of the working electrode which occurs with pulsing. This cleanup increases the electrode response.

Table 1. Residual Current Change, ΔI, and Decay Current, i_{decay}, in DC, DP and NP Voltammetry in Isocratic Elution. Flow rate: 1 mL/min; column: Altex Ultrasphere ODS 150 × 4.6 mm; E_i = 0.55 V, E_f = 1.30 V vs. Ag/AgCl; working electrode: glassy carbon; low pass filter = 0.3 sec; pulse amplitude = 100 mV.

Solvent A = 0.04 M $NaClO_4$ in water.
Solvent B = 0.04 M $NaClO_4$ in acetonitrile.

Figure	Mobile Phase	Electrochemical Mode	Scan Rate mV/sec
2-a	5% B	DC	2
2-b	5% B	DP	2
3-a	50% B	DC	2
3-b	50% B	DC	5
3-c	50% B	DP	2
4-a	95% B	DC	2
4-b	95% B	DP	2
5	95% B	NP	2

Table 1 - Extended

Drop Knocker Time (sec)	$\Delta I \pm$ Measurement Error (μA)	$i_{decay} \pm$ measurement Error (μA)
1	0.75 ± 0.02	0.16 ± 0.02
1	± 0.02	± 0.02
1	0.65 ± 0.01	0.12 ± 0.01
1	0.82 ± 0.01	0.34 ± 0.01
1	± 0.01	± 0.01
0.5	1.41 ± 0.02	0.10 ± 0.02
0.5	± 0.02	± 0.02
0.5	7.36 ± 0.08	± 0.08

Effect of Background Electrolytes and Gradient Elution on Baseline Shift Using Amperometric Detection

In isocratic elution, the composition of the mobile phase during the course of separation is constant; however, isocratic elution sometimes is incapable of separating complex samples which have a wide range of retention times or k' values. One of the solutions to this problem is gradient elution (GE). The necessity (of the presence) of a background electrolyte and changing composition of the mobile phase causes changes in viscosity, dielectric constant, and conductivity of the mobile phase. The dependence of the diffusion coefficient on viscosity, variation of the conductivity, and changing condition of the working electrode surface during gradient elution will cause a change in the residual current.

Walden's rule [40] states "the product of the equivalent conductivity (λ) and viscosity of the solvent (η) for a particular electrolyte at a given temperature should be a constant."

$$\lambda * \eta = \text{constant} \qquad (9)$$

In a binary gradient (solvents A and B), the percentage of organic modifier (solvent B) increases in hydroorganic mobile phase during the gradient course. Decreasing the polarity of the mobile phase during the gradient course decreases the conductivity of the mobile phase if the concentration of background electrolyte in both solvents is

the same. If the concentration of background electrolyte in solvent B is higher than that of solvent A, the total concentration of background electrolyte in mobile phase increases during the gradient course. An increase in the concentration of background electrolyte during the gradient course may compensate for decreasing the polarity and changing the viscosity of the mobile phase, which are important factors in baseline stability. As a result of this discussion, a more stable baseline should be achieved with higher concentration of background electrolyte in solvent B as compared to equal concentration of background electrolyte in both solvents.

If one assumes a binary gradient in which the percentage of solvent B increases during the gradient course, one can measure the conductivity or specific conductivity vs. the percent of solvent B in A+B. The specific conductivity vs. the percentage of organic modifier (solvent B) is shown in Figure 6 for H_3PO_4 and $NaClO_4$ as background electrolytes. The conductance measurement was done with a conductance bridge of the Janz-McIntyre type [41]. The specific conductivity, K, was calculated from using equation (10).

$$K = \text{specific conductivity} = \frac{0.126}{R} \qquad (10)$$

The cell constant is 0.126 cm^{-1}, and R can be calculated from equation (11).

$$R = \frac{R_{measured} \times 10000}{10000 - R_{measured}} \qquad (11)$$

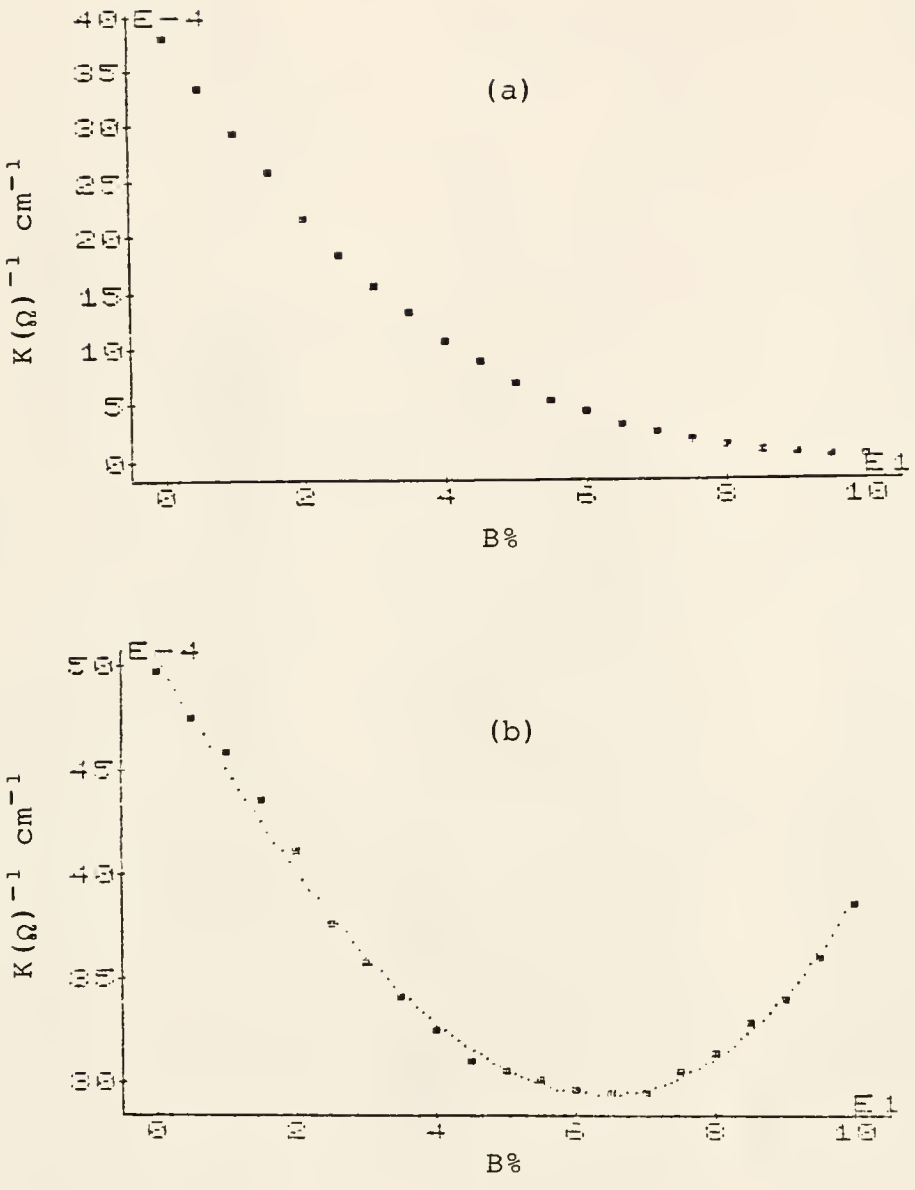


Figure 6. Specific Conductance vs. Percentage of Solvent B

(a) Solvent A = 0.15% H_3PO_4 in H_2O
Solvent B = 0.20% H_3PO_4 in CH_3OH

(b) Solvent A = 0.05 M $NaClO_4$ in H_2O
Solvent B = 0.05 M $NaClO_4$ in CH_3OH

In the case of H_3PO_4 as a background electrolyte, the specific conductance decreases with the increasing percentage of the organic modifier, while in the case of $NaClO_4$ it passes through a minimum at about 65% of organic modifier (solvent B).

Figure 7 shows the baseline shift during gradient elution (with no sample injection) using an amperometric detector and glassy carbon electrode, with H_3PO_4 and $NaClO_4$ as the background electrolytes. The column is equilibrated with solvent A, and gradient starts with holding solvent A for 3 min, then linear ramp to 20% A and 80% B over 30 min. The baseline shift, Δi, is the difference in current before starting the gradient program and at the end of the gradient. Figure 7-a is due to 0.15% H_3PO_4 in H_2O and 0.20% H_3PO_4 in CH_3OH, with Δi = -9 n A, while Figure 7-b is due to 0.20% H_3PO_4 in both H_2O and CH_3OH with Δi = -14 n A. The difference in Δi for Figures 7-a and 7-b is due to the concentration difference of H_3PO_4 in H_2O. The lower baseline shift in Figure 7-a as compared to Figure 7-b is in agreement with Walden's rule as discussed above. Figure 7-c is due to 0.05 M $NaClO_4$ in both H_2O and CH_3OH, with Δi = +6 n A. Sodium perchlorate produces a smaller baseline shift because it is completely ionized in both solvents. The peaks in Figure 7 might be due to impurities in H_2O or background electrolytes which collect at the top of the column during the early part of the solvent program and equilibration. More examples of gradient elution with hydroorganic and

Figure 7. Baseline Shift during Gradient Elution with No Sample Injection, Using an Amperometric Detector

Gradient Program: 100% A for 3 min, then linear ramp to 20% A, 80% b over 30 min

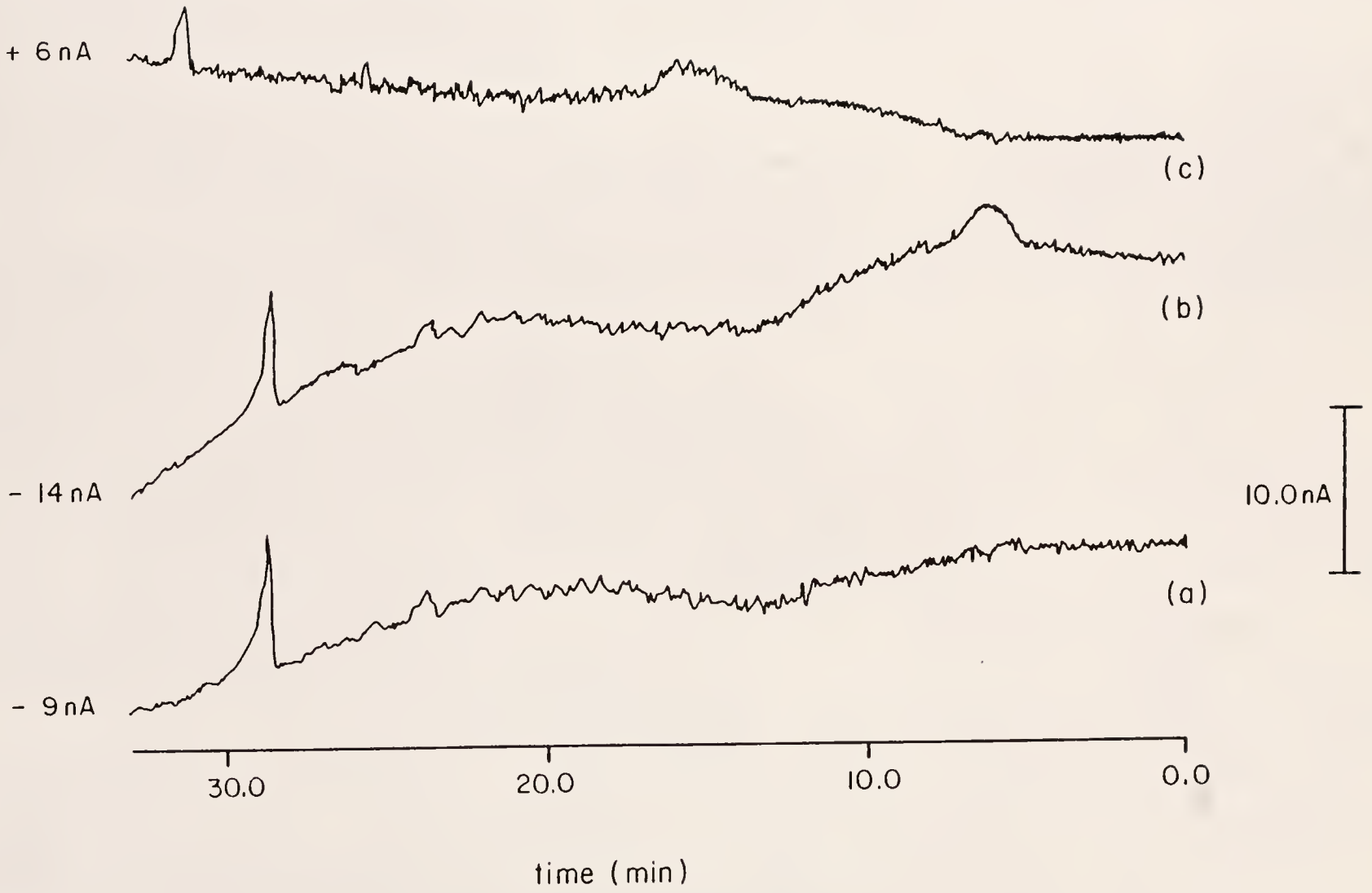
Flow rate: 2 mL/min; temperature: 30°C; working electrode: glassy carbon; E = 1.0 V vs. Ag/AgCl

Column: Altex Ultrasphere ODS 150 × 4.6 mm

(a) Solvent A = 0.15% H_3PO_4 in H_2O
Solvent B = 0.20% H_3PO_4 in CH_3OH

(b) Solvent A = 0.20% H_3PO_4 in H_2O
Solvent B = 0.20% H_{3PO_4} in CH_3OH

(c) Solvent A = 0.05 M $NaClO_4$ in H_2O
Solvent B = 0.05 M $NaClO_4$ in CH_3OH


+ 6 nA
(c)
(b)
- 14 nA
10.0 nA
(a)
- 9 nA
30.0
20.0
10.0
0.0
time (min)

micellar mobile phases are given in Chapters Five and Six.

The shift in baseline during gradient elution at a constant applied potential might be due to different concentrations of electroactive impurities, changing diffusion coefficient of impurities with a change in the viscosity, exhibition of different half-wave potential for specific electroactive components, and effect of organic modifier on the surface of the working electrode.

Effect of the Electrode Material on Baseline Shift Using Amperometric and NRDP Voltammetry Methods

The baseline shift during gradient elution is evaluated for glassy carbon and porous membrane gold electrodes. The electrochemical methods used were amperometric and non-ramping differential pulse (NRDP) voltammetry. In NRDP method, the difference in current sampled at the end of the pulse and shortly before the rise of the pulse is recorded. Since the pulse time is very short, there is virtually no change in the mobile phase composition with gradient elution during one pulse. The normalized baseline shift (current difference at the end and the start of gradient program divided by the surface area of the working electrode) for amperometric and NRDP voltammetry with their chromatographic conditions are reported in Table 2.

Data in Table 2 show that the absolute amount of the baseline shift in the amperometric method is much higher for gold electrode as compared to glassy carbon electrode.

Table 2. Normalized Baseline Shift (nA/mm^2) during Gradient Elution with No Sample Injection. Column: Altex 150×4.6 mm Ultrasphere ODS; E = 0.8 V vs. Ag/AgCl; pulse amplitude = 100 mV; low pass filter = 3 sec. Gradient program: initially 95% A, then linear ramp to 5% A over 20 min, and finally holding for 5 min at 5% A.

Solvent A = 0.20% H_3PO_4 in H_2O.
Solvent B = 0.20% H_3PO_4 in CH_3OH.

Electrochemical Method	Working Electrode	Normalized Baseline Shift nA/mm^2
Amperometric	Glassy Carbon	-1.5
NRDP	Glassy Carbon	-116.0
Amperometric	Gold	-356.7
NRDP	Gold	-152.8

This indicates that the residual current change with gradient elution on the gold electrode surface is larger as compared to glassy carbon electrode, which shows the gold is not an appropriate working electrode under these conditions. Although the absolute amount of the baseline shift in the case of NRDP is also larger for the gold electrode as compared to glassy carbon electrode, this gap is not as large as in the case of amperometric method. This might be due to cleanup of the electrode surface with pulsing.

CHAPTER FIVE

A COMPARISON OF MICELLAR AND HYDROORGANIC MOBILE PHASES USING AMPEROMETRIC DETECTOR

In this chapter, the following will be considered: (1) the comparison of the hydrodynamic voltammograms (HDV) in micellar and hydroorganic mobile phases, (2) the comparison of analytical figures of merit between micellar and hydroorganic mobile phases, and (3) gradient elution and selctivity with micellar mobile phases.

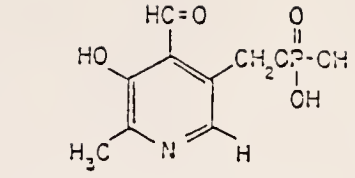
Ionic sufractants have been extensively used as ion-pairing reagents in ion-pair chromatography in the past. More recently, ionic surfactants and nonionic surfactants in aqueous solution have been employed as the mobile phase in RP-LC. The surfactant concentrations in the mobile phase above the CMC have been shown to have properties similar to conventional mobile phases for RP-LC [42]. Micellar solutions have been used as media or matrices for room temperature phosphorescence [43], and the usefulness of micelle-stabilized room-temperature phosphorescence (MSRTP) for detection and quantification of aromatic molecules in HPLC has been reported by Weinberger et al. [44]. Micellar solutions have been used in electrochemistry to produce well-defined redox waves for compounds that show only slight shoulders or no waves in aqueous solutions [45]. Since the

redox voltammetry waves for organic compounds in micellar solutions can be different from those of hydroorganic solutions, three different classes of compounds were chosen in this study. The structures of these three classes which include various forms of B-6 vitamins (PLP,PL, PN, PNP and PMP), phenolic compounds (phenol, hydroquinone, resorcinol, catechol and o-cresol), and polyaromatic hydrocarbons (anthracene and pyrene) are shown in Figure 8. The phosphate group in three of the B-6 vitamins can be partially ionized. The extent of ionization is dependent upon the pH of the mobile phase, however, in the low pH region, all forms of B-6 vitamins will be protonated to produce cations. A phosphoric acid buffer solution of pH 2.20 was used as the background electrolyte in all mobile phases and in addition generated an ionic site for ion-pairing of the nitrogenous vitamins. With cationic solutes, sodium dodecyl sulfate (SDS) in the mobile phase can act as an ion-pairing reagent both below and above the CMC.

Hydrodynamic Voltammogram in Micellar and Hydroorganic Mobile Phases

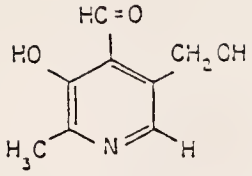
Hydrodynamic voltammograms for phenol, two vitamins (PLP and PL), and polyaromatic hydrocarbons (anthracene and pyrene) with their experimental conditions are given in Figures 9 through 11, respectively.

The signal achieved for the same amount of injected phenol in the micellar mobile phase is greater than that of the hydroorganic mobile phase when the applied potential is



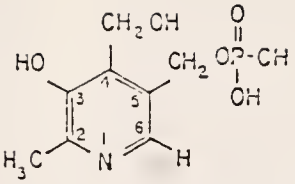
Pyridoxal-5-phosphate

PLP



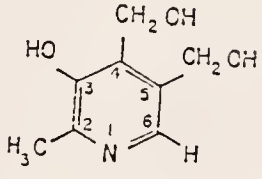
Pyridoxal

PL



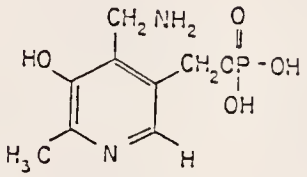
Pyridoxine-5-phosphate

PNP



Pyridoxine

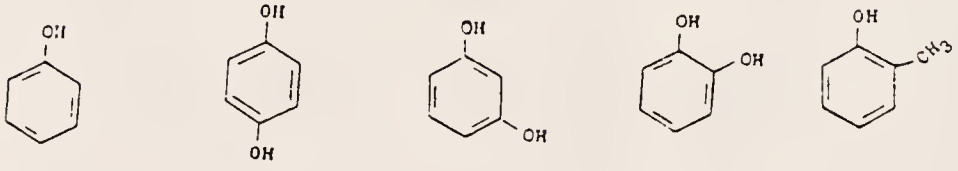
PN



Pyridoxamine-5-phosphate

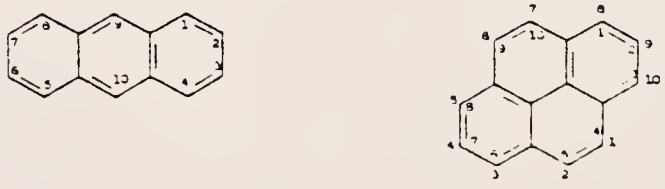
PMP

Structures of B-6 Vitamins



Phenol Hydroquinone Resorcinol catechol o-Cresol

Structures of Phenolic Compounds



Anthracene Pyrene

Structures of Polyaromatic Hydrocarbons

Figure 8. Chemical Structures of Compounds Used in Chapter Five

Figure 9. Hydrodynamic Voltammogram for Phenol

Column: Altex Ultrasphere ODS, 150 × 4.6 mm
Flow Rate: 1 mL/min
Injection Volume: 20 µL (5 ppm)
Working Electrode: Glassy carbon electrode
Temperature: 30°C

(o) Micellar mobile phase: 0.1 M SDS in (3:97) (1-propanol:H_2O) + 5 × 10^{-2} M $NaClO_4$

(•) Hydroorganic mobile phase: (60:40) (H_2O: CH_3OH) + 5 × 10^{-2} M $NaClO_4$

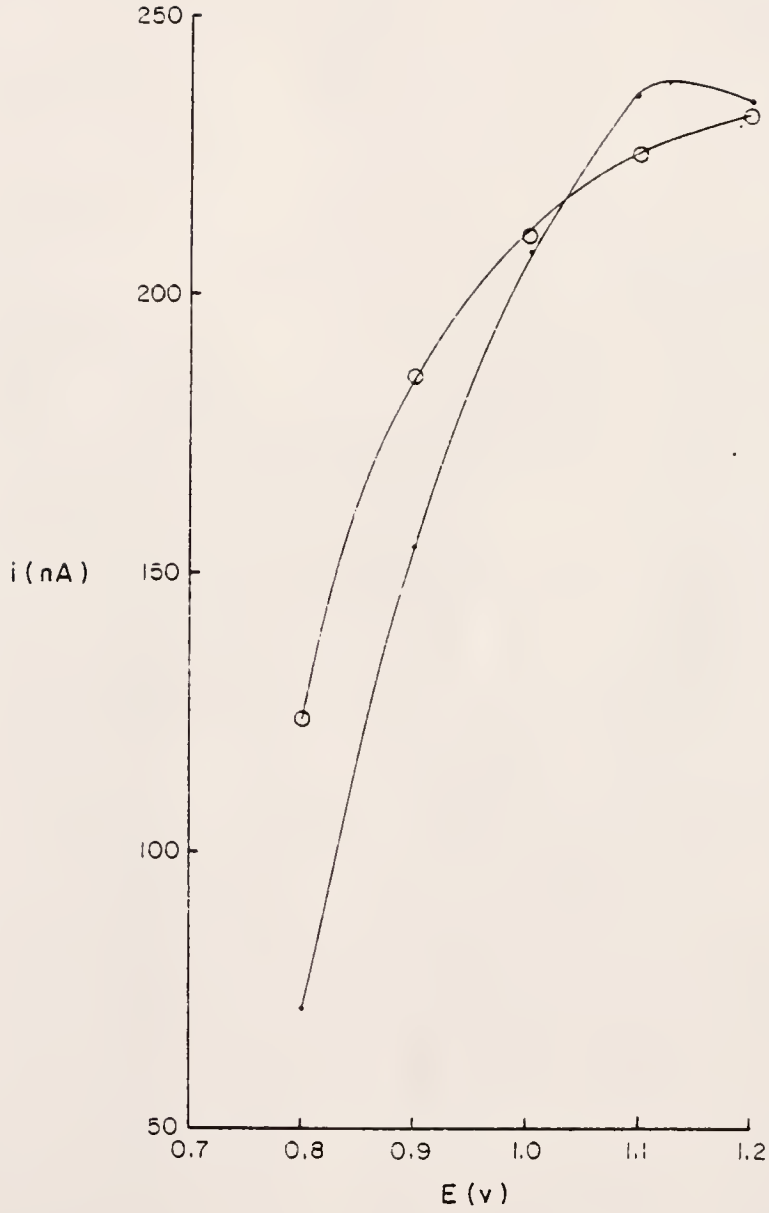

250
200
150
100
50
i (nA)
0.7
0.8
0.9
1.0
1.1
1.2
E (v)

Figure 10. Hydrodynamic Voltammogram for B-6 Vitamins[a]

Micellar mobile phase: 0.1 M SDS in (3:97) (1-propanol:H_2O), pH = 2.20 (used H_3PO_4)

(Δ) PLP (250 ppm)

(□) PL (250 ppm)

Ion-pair mobile phase: 5×10^{-3} M SDS in (12:88) (1-propanol:H_2O), pH = 2.20 (used H_3PO_4)

(o) PLP (2000 ppm)

(•) PL (2000 ppm)

---

a All B-6 vitamins dissolved in 0.1 M HCl. Other conditions as in Figure 9.

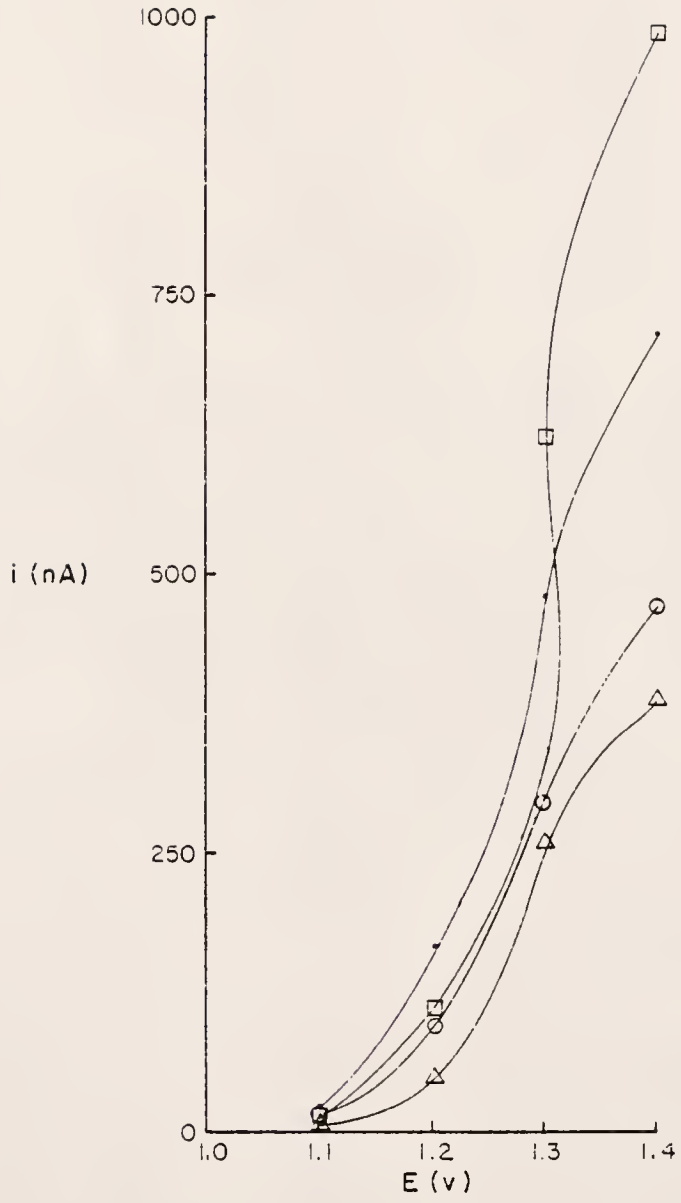

1000
750
500
250
0
i (nA)
1.0
1.1
1.2
1.3
1.4
E (v)

Figure 11. Hydrodynamic Voltammogram for Polyaromatic Hydrocarbons[a]

Column: Rainin microsorb octyl

Flow Rate: 2.5 mL/min

Micellar mobile phase: 0.1 M SDS in (3:97) (1-propanol:H_2O), pH = 2.2 (used H_3PO_4)

(o) anthracene (50 ppm)

(·) pyrene (100 ppm)

Hydroorganic mobile phases:

(□) anthracene (50 ppm): (46.5:53.5) (H_2O:CH_3OH), pH = 2.20 (used H_3PO_4)

(Δ) pyrene (100 ppm): (43.2:56.8) (H_2O:CH_3OH), pH = 2.20 (used H_3PO_4)

---

a Anthracene and pyrene were dissolved in CH_3OH. Other conditions as in Figure 9.


500
400
300
200
100
0
i (nA)
0.7
0.8
0.9
1.0
1.1
1.2
1.3
1.4
E (v)

below 1.0 V, whereas above 1.0 V it is slightly greater in the case of the hydroorganic mobile phase. According to HDV, the best analytical potential for phenol is 1.1 V in both mobile phases.

In the case of B-6 vitamins, the signal increases with increasing potential in the same manner for both the micellar mobile phase and the hydroorganic mobile phase which contains small amounts of SDS as an ion-pairing reagent. The signal for PLP and PL are greater in the micellar mobile phase as compared with the ion-pairing mobile phase. At first glance, the graph in Figure 10 shows that the signal for PLP in the ion-pairing mobile phase is greater than that in the micellar mobile phase at the same potential, however, this is not the case because the absolute amount of injection of PL and PLP in the ion-pairing mobile phase is 8 times greater than that in the micellar mobile phase. The potential 1.4 V might be chosen as an analytical potential for vitamins.

Figure 11 shows the HDV for anthracene and pyrene in both mobile phases. At potentials higher than 1.2 V, the signal due to pyrene increases sharply, whereas the signal due to anthracene decreases smoothly. One of the most important aspects of Figure 11 in comparison to Figures 9 and 10 is the unique selectivity response of polyaromatic hydrocarbons with applied potential in both micellar and hydroorganic mobile phases. This means that anthracene can be measured in the presence of pyrene using a proper applied potential. In order to compare analytical figures of merit

in micellar and hydroorganic mobile phases, the best possible analytical potential to use for anthracene and pyrene would be 1.1 V and 1.2 V, respectively.

Analytical Figures of Merit Comparison between Micellar and Hydroorganic Mobile Phases

Analytical figures of merit such as limit of detection (LOD), upper limit of linear dynamic range (LDR), sensitivity, correlation coefficient, and log-log slope, as well as the experimental conditions for phenol, B-6 vitamins, and polyaromatic hydrocarbons (PAH) in both micellar and hydroorganic mobile phases, are reported in Tables 3 through 5, respectively. Data in Tables 3 through 5 are derived from Figures 12 through 14 (analytical or calibration curves), respectively.

In order to compare analytical figures of merit for a specific electroactive component in two different mobile phase compositions using an amperometric detector, two constraints must be met. First, the flow rate should be nearly the same for both mobile phases because the response of any electrochemical detector is dependent on the rate of mass transfer to the electrode surface [37]. Secondly, the retention time of the electroactive component in both mobile phases should be nearly the same because of the peak height measurement. To get nearly the same retention time in both mobile phases, the percentage of organic modifier in the hydroorganic mobile phase was varied until the retention

Table 3. Analytical Figures of Merit for Phenol. Flow rate: 1 mL/min; column: Altex Ultrasphere ODS, 150 × 4.6 mm; E = 1.1 V; working electrode: glassy carbon electrode; injection volume: 20 µL.

Micellar mobile phase: 0.1 M SDS in 3% 1-propanol + 5×10^{-2} M $NaClO_4$.

Hydroorganic mobile phase: (H_2O:CH_3OH)(60:40) + 5×10^{-2} M $NaClO_4$.

	Limit of Detection		Upper limit of LDR	
Mobile Phase	(ppm)	(ng)	(ppm)	(ng)
Micellar	0.0080	0.16	20[a]	400
Hydroorganic	0.0065	0.13	10	200

a 20 ppm was the most concentrated solution, so the upper limit of LDR might be more than 20 ppm.

Table 3 - Extended

Sensitivity (nA/ppm)	Sensitivity (nA/ng)	Correlation Coefficient	Log-log Slope	t_R (min)
41.40	2.03	0.9971	0.96	5.0
39.74	1.99	0.9973	1.04	3.8

Table 4. Analytical Figures of Merit for B-6 Vitamins.
Column: Altex Ultrasphere ODS, 150 × 4.6 mm;
E = 1.4 V; working electrode: glassy carbon;
injection volume: 20 µL.

Micellar mobile phase: 0.1 M SDS in 3% 1-propanol with pH = 2.20 (used H_3PO_4).
Ion-pair mobile phase: 5×10^{-3} M SDS in 12% 1-propanol with pH = 2.20 (used H_3PO_4).

Compound	Mobile Phase	Limit of Detection (ppm)	Limit of Detection (ng)	Upper Limit of LDR (ppm)	Upper Limit of LDR (ng)
PLP	Micellar	0.316	6.32	300	6000
PL	Micellar	0.236	4.72	300	6000
PLP	Ion-pair	0.0726	1.45	100	2000
PL	Ion-pair	0.0852	1.70	100	2000

Table 4 - Extended

Sensitivity (nA/ppm)	Sensitivity (nA/ng)	Correlation Coefficient	Log-log Slope	Flow Rate (mL/min)	t_R (min)
3.80	0.170	0.9999	0.99	1.0	2.8
5.09	0.255	0.9995	1.00	1.0	7.8
4.96	0.248	0.9999	0.95	1.1	2.8
4.22	0.211	0.9999	0.95	2.0	7.8

Table 5. Analytical Figures of Merit for Polyaromatic Hydrocarbons. Flow rate: 2.5 mL/min; column: Rainin microsorb octyl, 150 × 4.6 mm; injection volume: 20 µL.

Micellar mobile phase: 0.1 M SDS in (3:97)(1-propanol-H_2O), pH = 2.20 (used H_3PO_4)

Compound	Mobile Phase	Limit of Detection (ppm)	Limit of Detection (ng)	Upper Limit of LDR (ppm)	Upper Limit of LDR (ng)
Anthracene	Micellar	0.0933	1.87	250	5000
Pyrene	Micellar	1.77	35.4	200	4000
Anthracene	Hydroorganic[a]	0.0846	1.69	50	1000
Pyrene	Hydroorganic[b]	--	--	--	--

a Hydroorganic mobile phase (used only for anthracene): (46.5:53.5)(H_2O:CH_3OH); pH = 2.20 (used H_3PO_4); ionic strength = 3.59×10^{-2} M.

b Hydroorganic mobile phase (used only for pyrene): (43.20:56.80)(H_2O:CH_3OH), pH = 2.20 (used H_3PO_4).

Table 5 - Extended

Sensitivity (nA/ppm)	Sensitivity (nA/ng)	Correlation Coefficient	Log-log Slope	E (V)	t_R (min)
0.707	0.0354	0.9996	0.97	1.1	13.7
0.667	0.0338	0.9990	0.97	1.2	9.3
3.545	0.177	0.9906	0.96	1.1	13.7
--	--	--	--	1.2	9.3

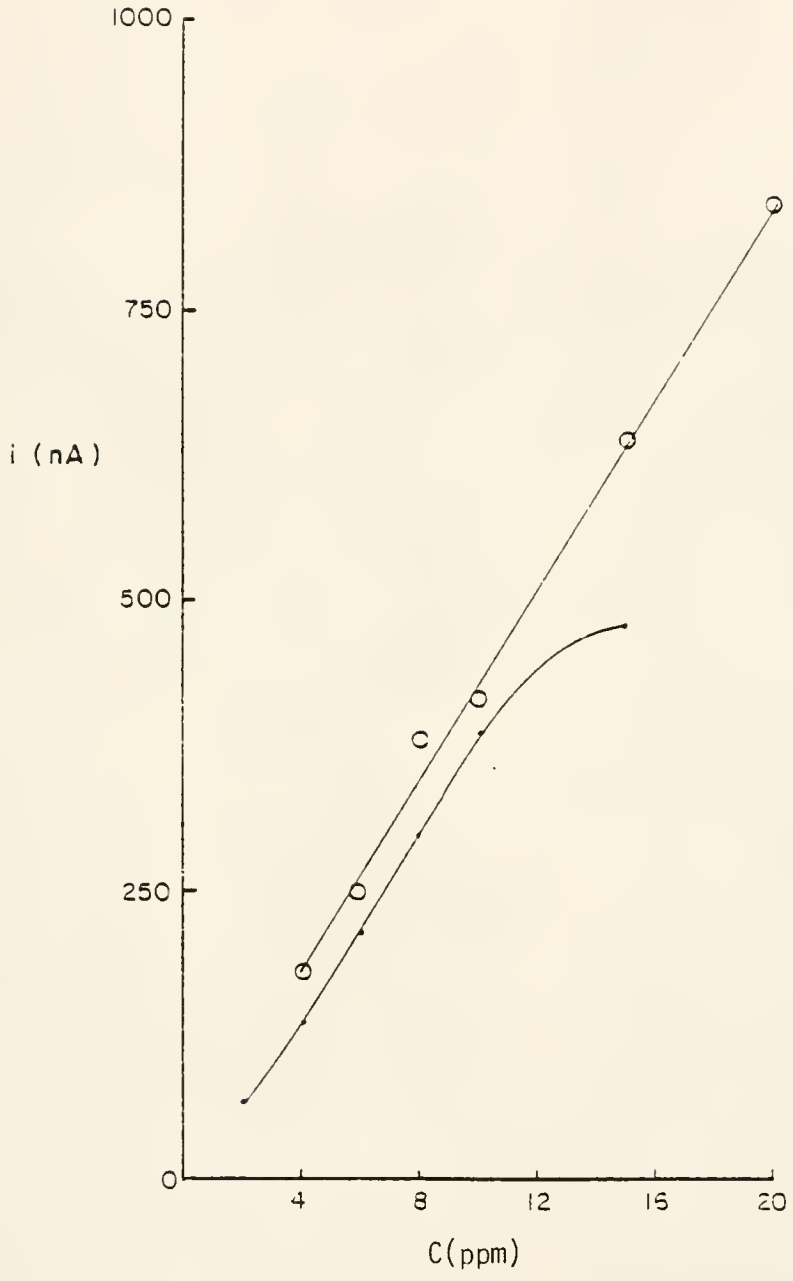


Figure 12. Analytical Curves for Phenol

(o) in micellar mobile phase
(•) in hydroorganic mobile phase

Experimental conditions are the same as Table 3.

Figure 13. Analytical Curves for B-6 Vitamins

Micellar mobile phase:

(Δ) PLP

(□) PL

Ion-pair mobile phase:

(o) PLP

(•) PL

Experimental Conditions are the same as Table 4.


2000
1500
1000
500
0
i (nA)
100
200
300
400
500
C(ppm)

Figure 14. Analytical Curves for Polyaromatic Hydrocarbons

Micellar mobile phase:

(o) anthracene

(•) pyrene

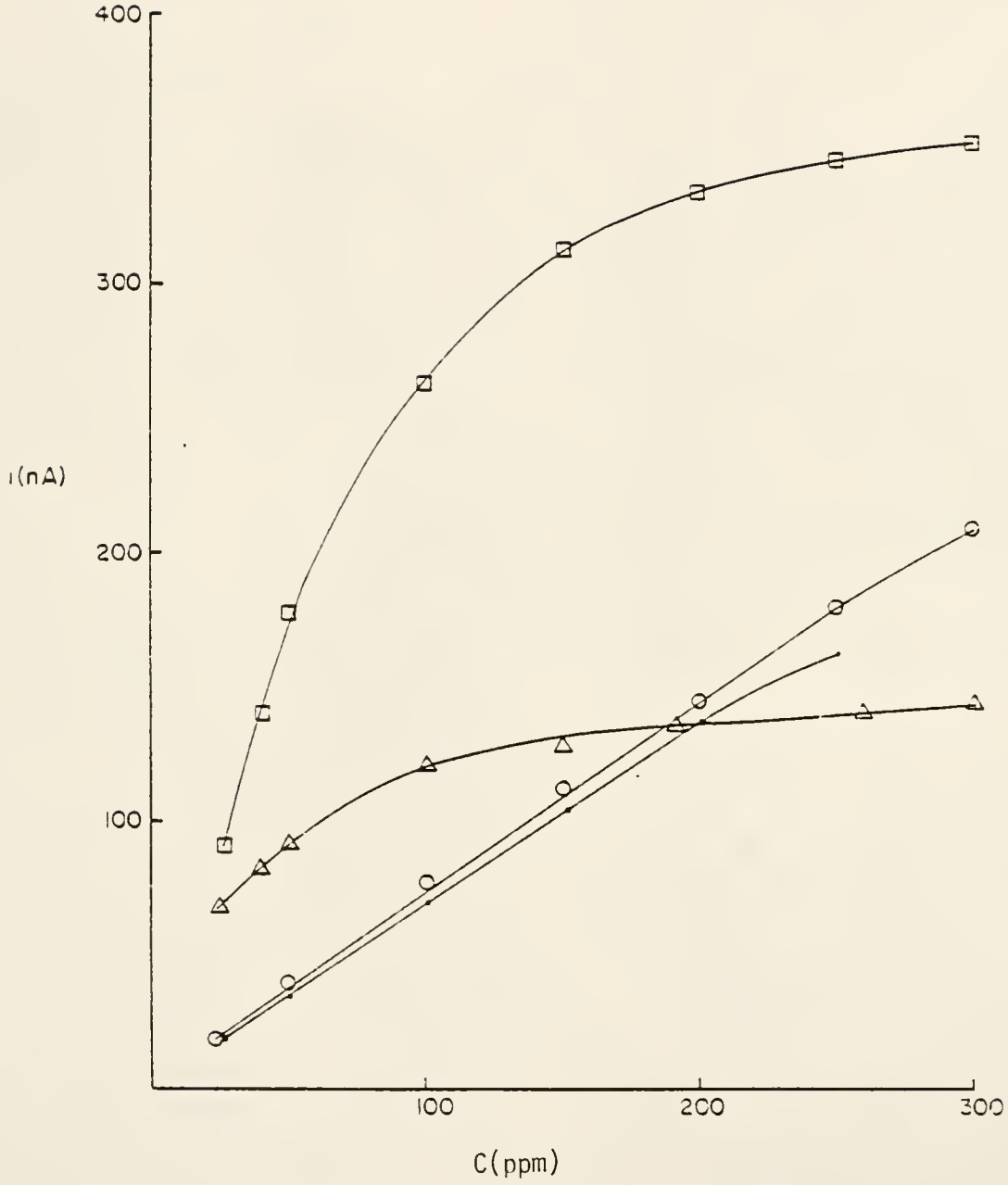
Hydroorganic mobile phase:

(□) anthracene

Hydroorganic mobile phase:

(△) pyrene

Experimental conditions are the same as Table 5.


400
300
200
100
I(nA)
100
200
300
C(ppm)

time of the electroactive component nearly matched that of the micellar mobile phase.

In the case of phenol, the LOD is approximately the same in both mobile phases, whereas the upper limit of the LDR in the micellar mobile phase is at least twice that of the hydroorganic mobile phase. For B-6 vitamins there is not much difference in the LOD in both mobile phases, while the upper limit of LDR in micellar mobile phase is 3 times that of the hydroorganic mobile phase. The LDR for anthracene is 5 times greater in the micellar mobile phase as compared to the hydroorganic mobile phases, while the LOD is approximately the same in both mobile phases. It is hard to determine the precise analytical figures of merit for pyrene in the hydroorganic mobile phase because, as Figure 14 shows, the analytical curve is practically non-linear in that concentration range. Pyrene, however, should be similar to anthracene, and, as shown in Figure 14, the upper limit of the LDR for the micellar mobile phase is much greater when compared to the hydroorganic mobile phase.

The greater upper limit of LDR in the micellar mobile phase as compared to the hydroorganic mobile phase may be due to the higher ionic strength (resulting from the presence of ionic surfactants as well as background electrolyte), in the micellar mobile phase [19,46] or due to the nature of surfactants. Increasing the ionic strength of the mobile phase decreases the IR gradient across the electrode face and between the electrode and the bulk solution. Surfactants or surface active agents can change the interfacial

properties. Hence, the interfacial properties of the micellar mobile phase with working electrode will be quite different from those of hydroorganic mobile phase.

Gradient Elution and Selectivity in Micellar Mobile Phase

Gradient elution with the micellar mobile phase is primarily used for the same purpose as it is with the hydroorganic mobile phase. Gradient elution may also be used to effect a change in selectivity because micellar mobile phases have unique selectivities at different concentrations. Armstrong and Henry [42] have used gradient flow rates with a UV detector. The gradient micellar chromatogram, obtained with an amperometric detector using an anionic surfactant (SDS) and a nonionic surfactant (Brij-35) are shown in Figures 15 through 17. Figure 15 shows the separation of B-6 vitamins with SDS, and Figures 16 and 17 show separation of phenolic compounds with SDS and Brij-35, respectively.

Micellar mobile phases have been shown [47] to offer control over selectivity in liquid chromatography as the concentration of surfactant changes in the mobile phase. The selectivity of the micellar mobile phase toward B-6 vitamins is shown in Figure 18. The elution order of PL and PMP changes above and below a certain concentration range of SDS, whereas they coelute in this range. The void volume of the column, determined by water injection, slightly decreased as the concentration of SDS increased in the mobile phase. For an Altex Ultrasphere ODS 25 × 4.6 mm, a

Figure 15. Gradient Micellar Chromatogram for Separation of Vitamin B-6

Solvent A: (3:97)(1-propanol:H_2O), pH = 2.20

Solvent B: 0.2 M SDS in (3:97)(1-propanol: H_2O), pH = 2.20 (used H_3PO_4)

Gradient program: started with 25% solvent B and held at 25% B for 8 min, then ramped to 100% B over 8 min and continued with 100% B

Flow rate: 1 mL/min; temperature: 30°C; working electrode: glassy carbon electrode; E = 1.3 V

Column: Altex Ultrasphere ODS, 250 × 4.6 mm; precolumn: 15 × 4.6 mm, packed manually with 25-40 μm silica gel

Injection volume: 10 μL

Peaks are as follows: (1) PLP (200 ppm), (2) PL (200 ppm), (3) PMP (400 ppm), (4) PNP (400 ppm), (5) PN (400 ppm)

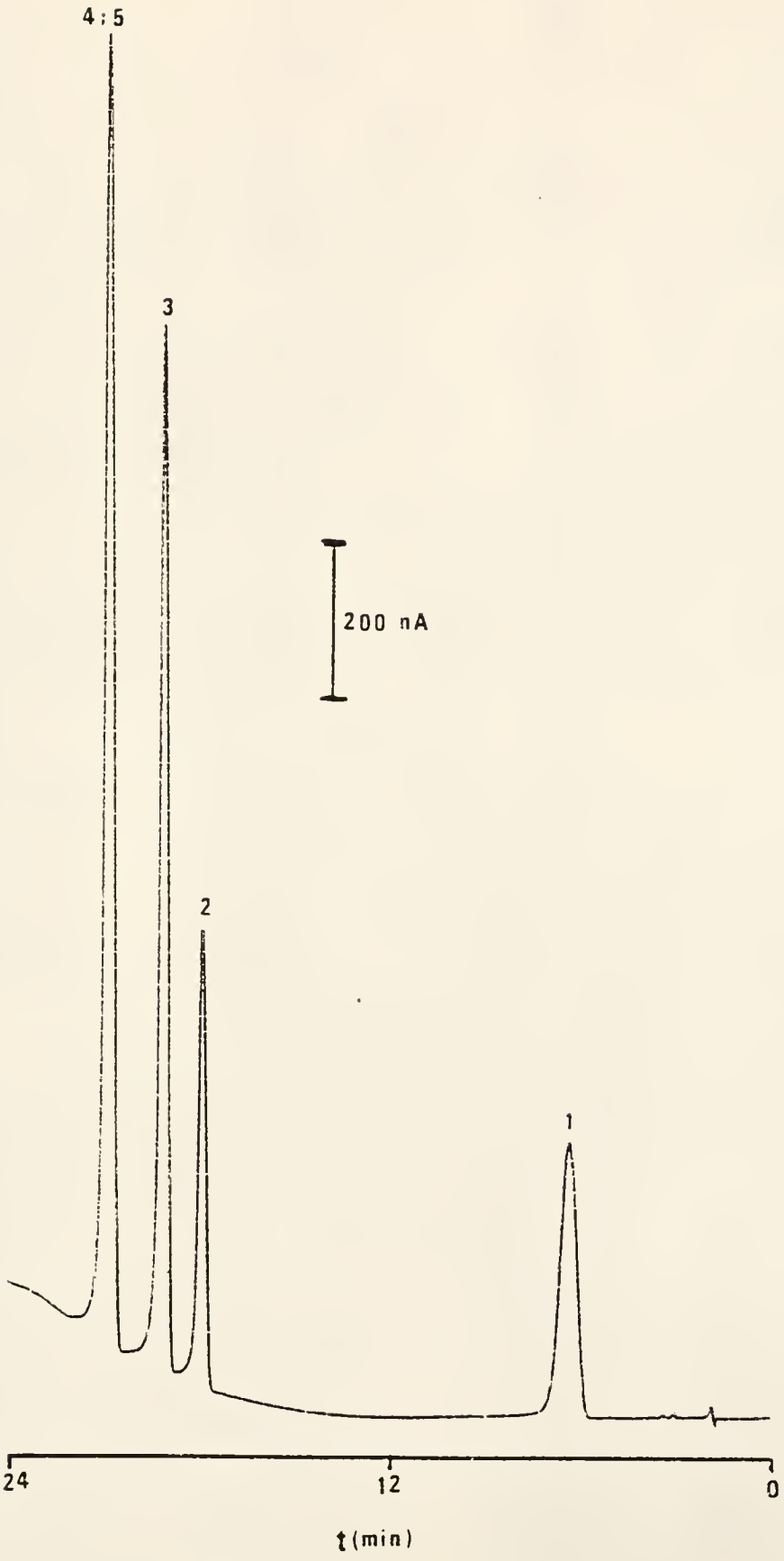

4:5
3
200 nA
2
1
24
12
0
t(min)

Figure 16. Gradient Micellar Chromatogram for Separation of Phenolic Compounds

Gradient program: started with 25% solvent B and held at 25% B for 4 min, then ramped to 100% B over 8 min and continued with 100% B

Injection volume: 13 μL; E = 1.1 V

Peaks are as follows: (1) hydroquinone (10 ppm), (2) resorcinol (8 ppm), (3) catechol (20 ppm), (4) phenol (18 ppm), (5) o-cresol (arbitrary concentration)

Other conditions as Figure 15.

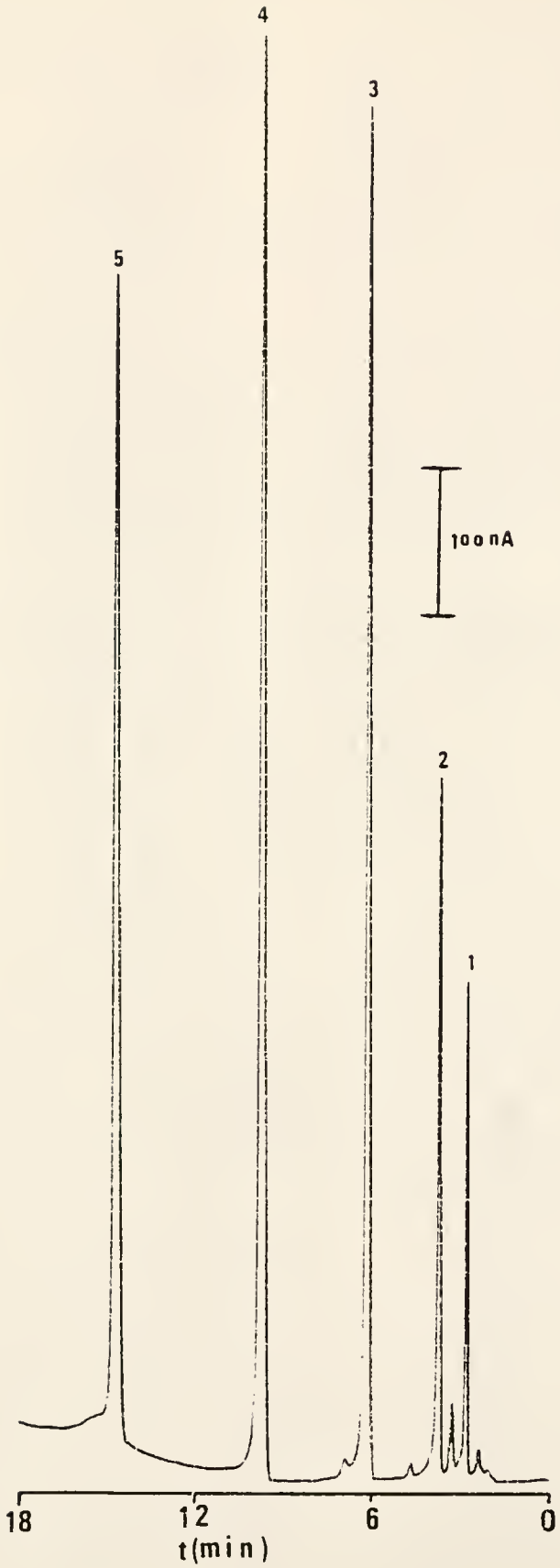

4
3
5
100nA
2
1
18
12
6
0
t(min)

Figure 17. Gradient Micellar Chromatogram for Separation of Phenolic Compounds and Gradient with No Injection

Solvent A: (3:97)(1-propanol:H_2O), pH = 2.20 (used H_3PO_4)

Solvent B: 0.1 M Brij-35 in (3:97)(1-propanol:H_2O), pH = 2.20 (used H_3PO_4)

Gradient program: started with 20% solvent B and held at 20% B for 4 min, then ramped linearly to 100% B over 6 min and continued with 100% B

Flow rate: 1 mL/min; temperature: 30°C; working electrode: glassy carbon electrode

Column: Altex Ultrasphere ODS, 150 × 4.6 mm

Injection volume: (a) 20 µL; (b) none

Peaks are as follows: (1) hydroquinone (10 ppm), (2) resorcinol (8 ppm), (3) catechol (20 ppm), (4) phenol (18 ppm), (5) o-cresol (arbitrary concentration); these are for (a)

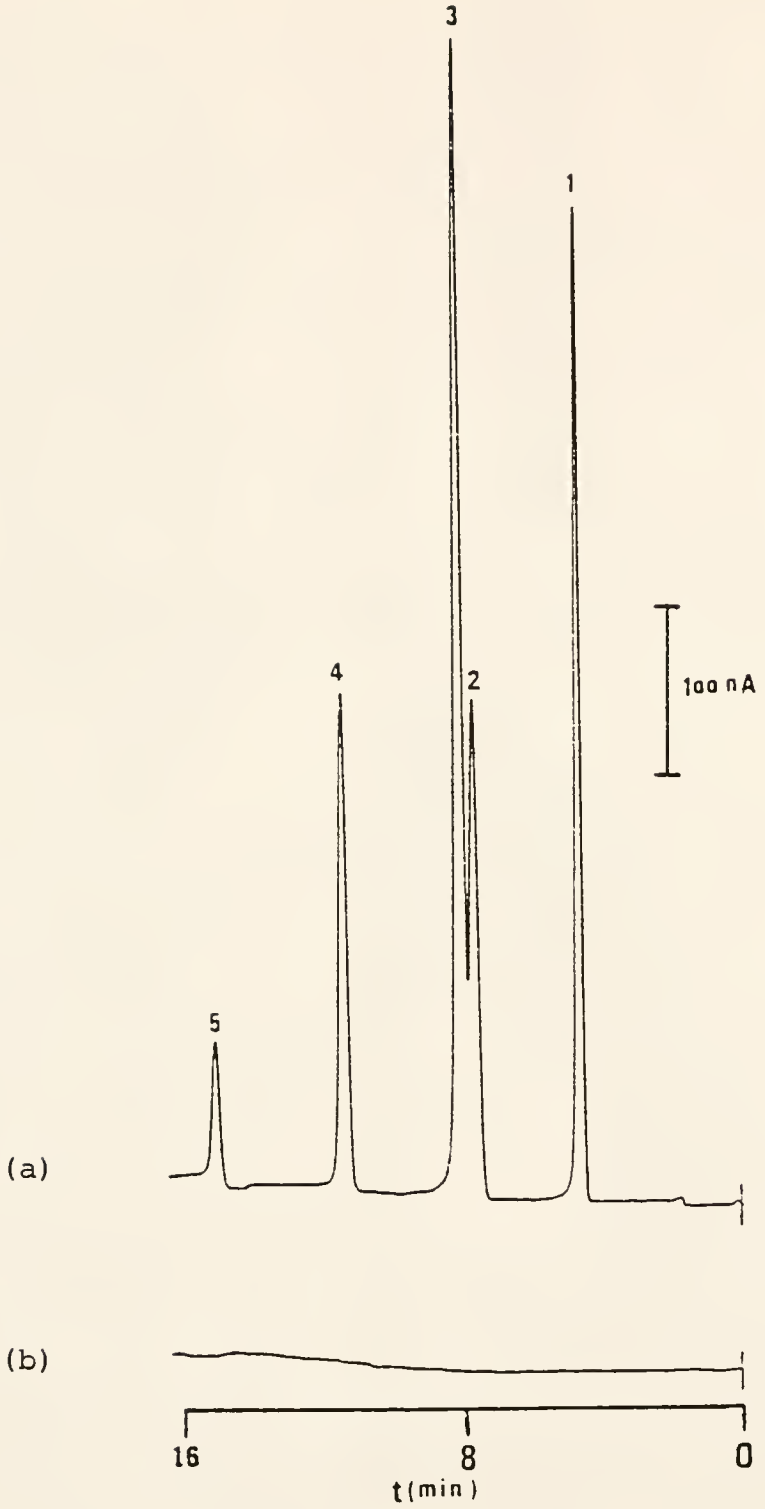

3
1
4
2
100 nA
5
(a)
(b)
16
8
0
t(min)

Figure 18. Effect of SDS Concentration on k'

Mobile phase: (3:97)(1-propanol:H_2O) with different concentrations of SDS, pH = 2.20 (used H_3PO_4)

Flow rate: 1 mL/min; temperature: 30°C; working electrode: glassy carbon; E = 1.3 V

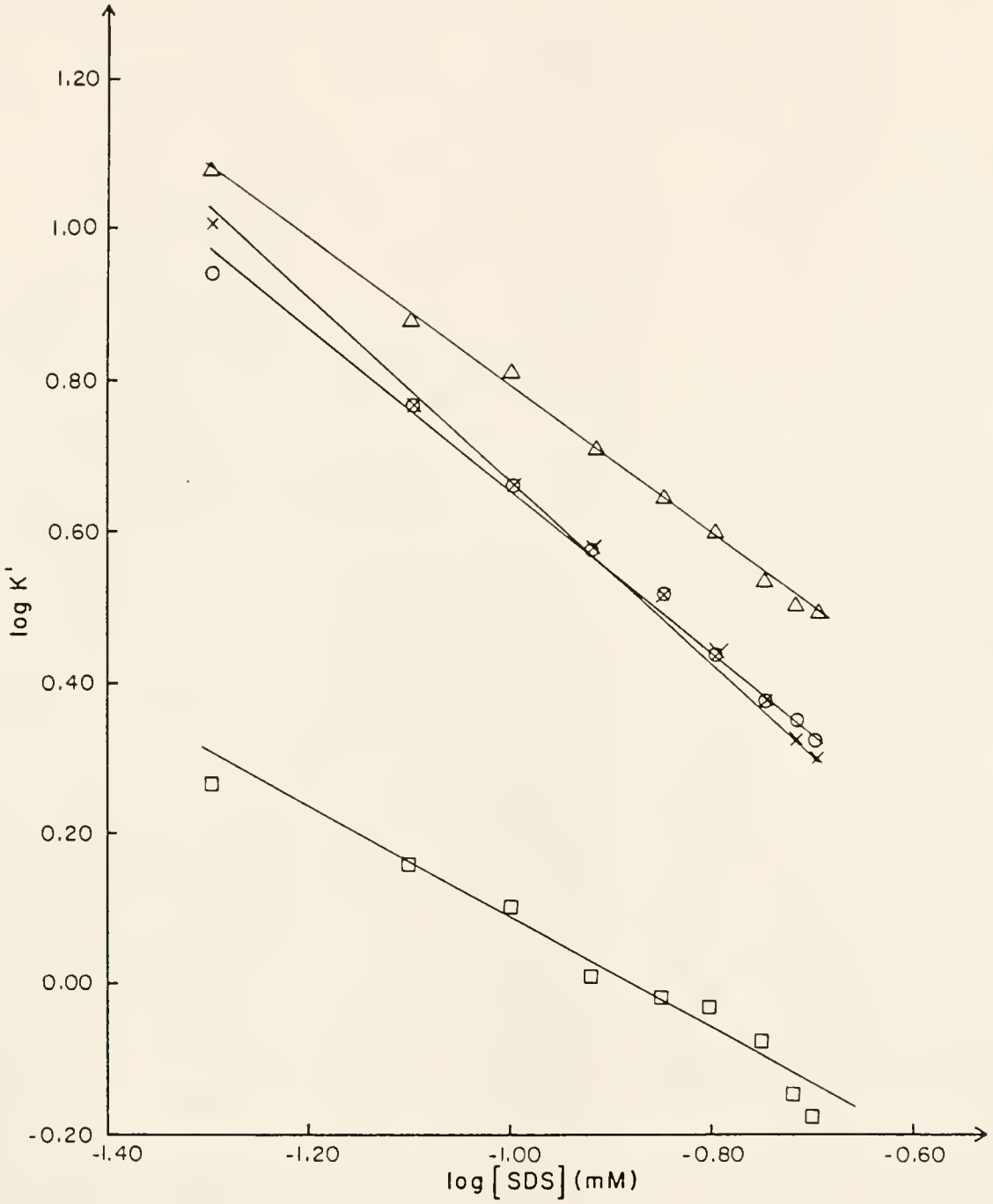
Column: Altex Ultrasphere ODS, 250 × 4.6 mm

(□) PLP

(o) PL

(×) PMP

(Δ) PN and PNP


1.20
1.00
0.80
0.60
0.40
0.20
0.00
-0.20
log K'
-1.40
-1.20
-1.00
-0.80
-0.60
log [SDS] (mM)

change in concentration of SDS from 0.05 M to 0.15 M or more in the mobile phase caused the void volume to change from 2.20 mL to 2.00 mL. The adsorption isotherm of surfactants on ODS-hypersil is reported by Knox and Hartwick [48]. The surfactant concentrations chosen were below the CMC and one SDS concentration supposedly above the CMC was also used. The adsorption isotherms shown by these researchers are in methanol:water (20:80) solution which results in a different CMC from that of pure water. It is doubtful they ever reached the CMC for their system. There is no literature adsorption isotherm available for SDS above the CMC. Figures 19 through 21 show the isocratic micellar chromatograms for B-6 vitamins and their selectivity with three different concentrations of SDS in the mobile phase.

Figure 19. Isocratic Micellar Chromatogram for Separation of Vitamin B-6

Mobile phase: 0.05 M SDS in (3:97)(1-propanol:H_2O), pH = 2.20 (used H_3PO_4)

Flow rate: 1 mL/min; temperature: 30°C; working electrode: glassy carbon electrode; E = 1.3 V

Column: Altex Ultrasphere ODS, 250 × 4.6 mm; precolumn: 15 × 4.6 mm, packed manually with 25-40 µm silica gel

Injection volume: 10 µL

Peaks are as follows: (1) PLP (200 ppm), (2) PL (200 ppm), (3) PMP (400 ppm), (4) PNP (400 ppm), (5) PN (400 ppm)

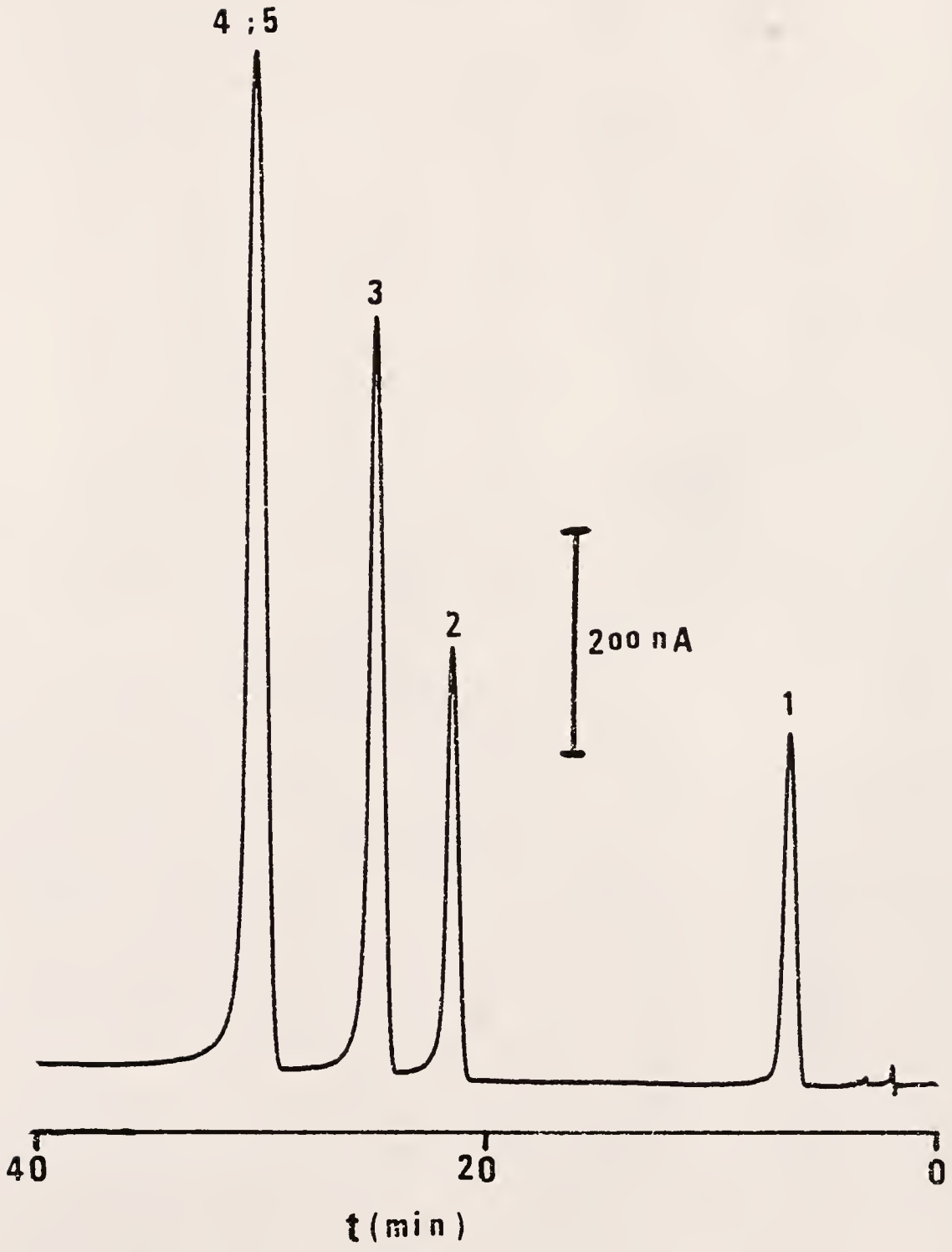

4 ; 5
3
2
200 nA
1
40
20
0
t (min)

Figure 20. Isocratic Micellar Chromatogram for Separation of Vitamin B-6

Mobile phase: 0.1 M SDS in (3:97)(1-propanol:H_2O), pH = 2.20 (used H_3PO_4)

Other conditions are as in Figure 19.

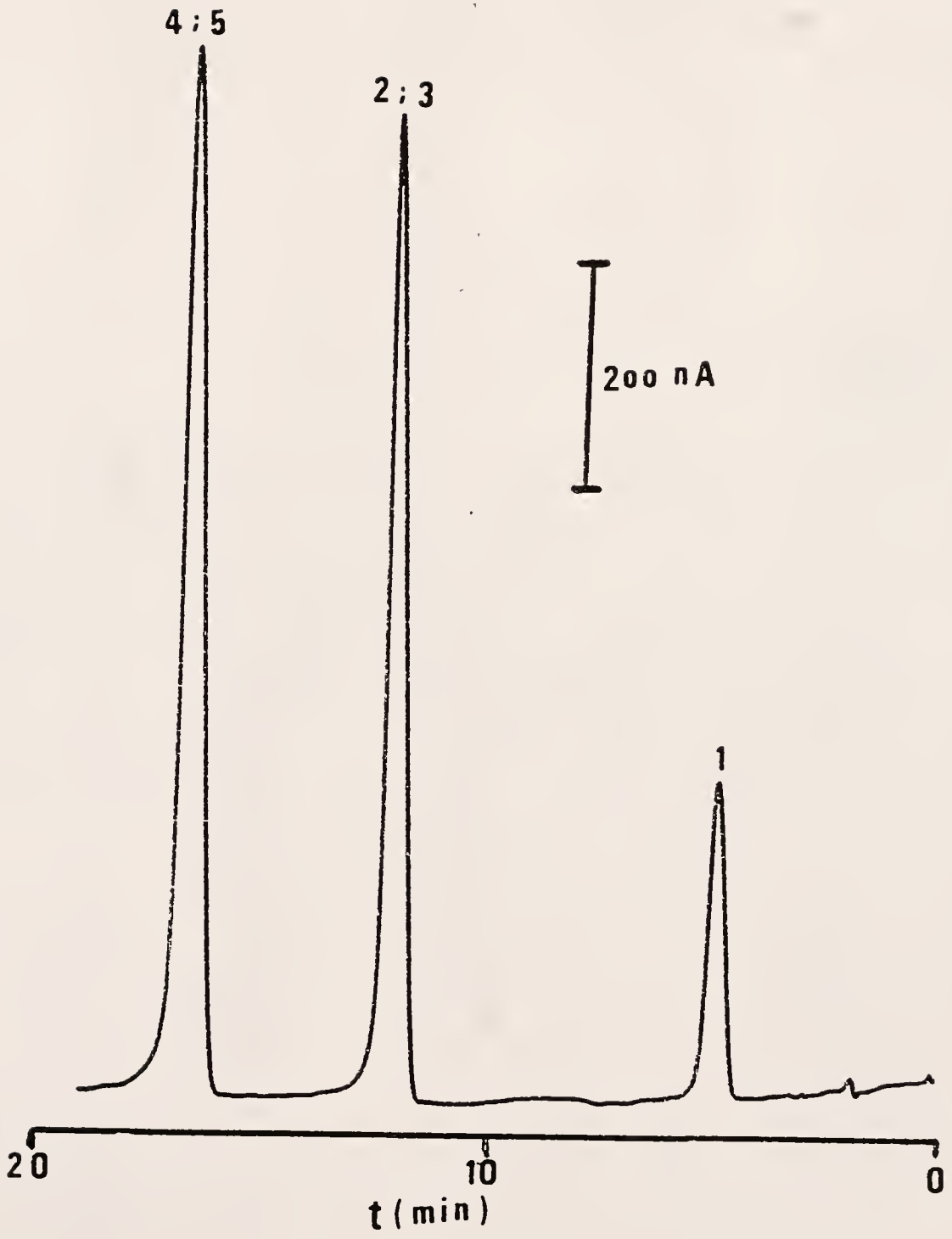
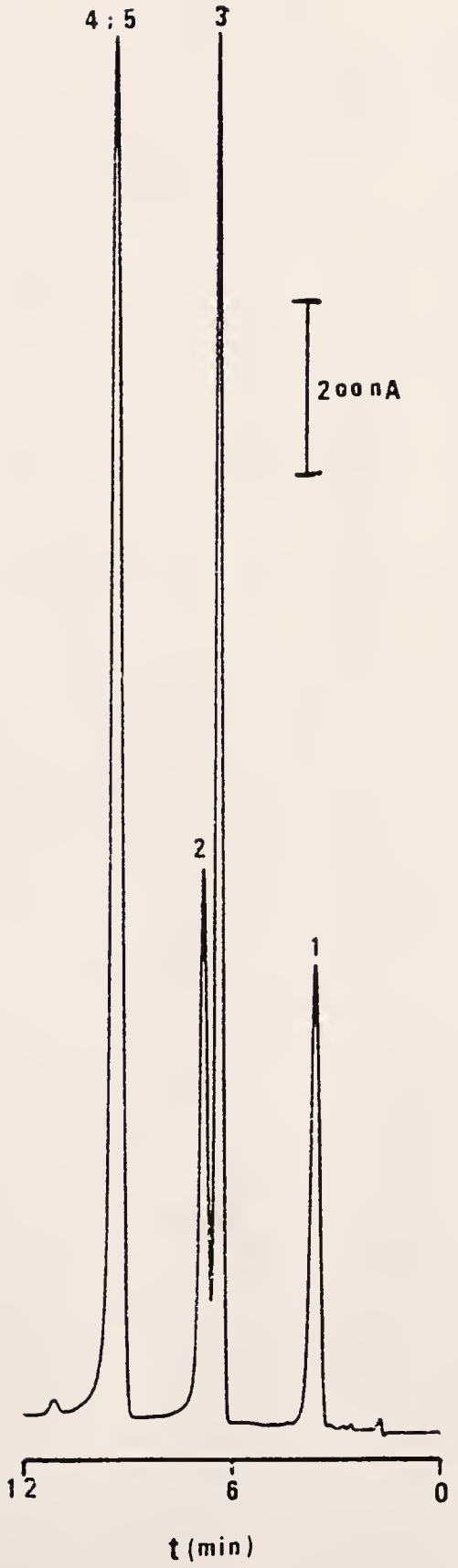

4 ; 5
2 ; 3
200 nA
1
20
10
0
t (min)

Figure 21. Isocratic Micellar Chromatogram for Separation of Vitamin B-6

Mobile phase: 0.2 M SDS in (3:97)(1-propanol:H_2O), pH = 2.20 (used H_3PO_4)

Other conditions are as in Figure 19.


4 ; 5
3
200 nA
2
1
12
6
0
t (min)

CHAPTER SIX

RAPID SEPARATION AND DETERMINATION OF THYROMIMETIC IODOAMINO ACIDS BY GRADIENT ELUTION REVERSE PHASE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

The mechanism of action of the thyroid hormones, T_3 and T_4, is of considerable interest in part because of the amazing diversity of thyroid hormone effects. These agents influence the metabolism of almost every class of foodstuff. They exert profound effects on many enzymes and on almost all organ systems, and they play an integral role in the complex biological processes involved in growth and differentiation [49]. The time-consuming procedures in USP monographs have been used for analysis of levothyroxine sodium tablets [50] and liothyronine sodium tablets [51]. The assay of the major thyroid hormones T_3 and T_4 also is done by wet analysis [52-54], radioimmunoassay [55-58], chemical derivatization followed by gas chromatography with electron capture detection [59,60], thin-layer chromatography [61-63], paper chromatography [64,65], electrophoresis [66], and gas chromatography/mass spectrometry [67]. Thin-layer and paper chromatography as well as the electrophoretic procedures do not have good limits of detection for this kind of analysis. Although the gas chromatographic procedures are sensitive, they require the isolation of the iodoamino acids in a pure form, which must then be converted to a volatile derivative

for chromatographic analysis. The radioimmunoassay procedures are impractical for a small number of samples and have the added problem of disposal of the radioactive wastes.

Liquid chromatography would normally be the method of choice for these analyses because of the poor volatility of the compounds. In fact, HPLC methods for separation of pure iodoamino acids have appeared in the literature [68-72]. Recently, T_3 and T_4 tablets have been analyzed by reverse phase LC using UV detection [73], and a gradient elution separation of sixteen thyromimetic iodoamino acids has been reported [74]; however, the low molar absorptivity of these compounds at 254 nm precludes their determination at trace levels. Detection at 220 nm improves this situation somewhat [74], and a clever catalytic post-column detection scheme has also been shown [75]. Most recently, application of amperometric electrochemical detection to these compounds has been shown to give excellent limits of detection [76], as has dansyl derivatization and subsequent fluorescence detection [77].

The purpose of this study was to demonstrate the usefulness of gradient elution techniques with electrochemical detection for the separation of seven thyromimetic iodoamino acids. A rapid isocratic separation of T_0, T_2, T_3 and T_4, as well as analysis of T_4 both in tablets and injectable intravenous samples is presented.

Standard Solutions

The compounds Tyr, MIT, DIT, T_0, T_2, T_3 and T_4 were purchased from Sigma Chemical (St. Louis, Missouri) and were stored in a freezer. Standard solutions were prepared by dissolving appropriate amounts of each compound in methanol containing 1% ammonium hydroxide and were stored in a refrigerator.

Preparation of T_4 Tablet Solution and Injectable T_4 Sample

Twelve tablets (1.5676 g) containing levothyroxine sodium were dissolved in 20 mL of 0.01 M sodium hydroxide using an ultrasonic bath. The sample solution was heated at 60°C for 3 min, shaken for 3 min, and then filtered through F2406-9 (S/P) filter paper. Before chromatographic injection, this solution was again filtered with a Rainin (Rainin Instruments, Woburn, Massachusetts) HPLC sample filter syringe using a 0.45 μm nylon-66 membrane filter. The injectable sample was present as a powder and was prepared by dissolving in 5 mL 0.9% sodium chloride solution. This resulted in a clear solution which was then filtered with the sample filter syringe.

Gradient Elution LC/EC

Amperometric electrochemical detectors are generally considered incompatible with gradient elution

techniques [78,17]. The necessity of the presence of a background electrolyte and the dependence of the charging or residual current on the exact composition of the mobile phase has discouraged attempts to use this powerful liquid chromatographic technique. Changes in the polarity and dielectric constant of the mobile phase during a gradient program yield steeply sloping baselines from the ever changing charging current. Indeed, to our knowledge, the only published report of gradient elution LC/EC used a gradient of only 36-60% methanol [79].

Addition of phosphoric acid to both constituents of the mobile phase (H_2O and CH_3OH) provides background electrolyte and suppresses the ionization of the thyromimetic iodoamino acids [80]. Both mobile phase constituents were degassed with helium gas during chromatographic runs. Initial attempts were made using equal concentrations of background electrolyte in both the water and methanol reservoirs. During a gradient from 0 to 100% methanol, a large negative shift in background was noted, so the background electrolyte concentration was increased in the methanol reservoir. This then increased the concentration of background electrolyte in the mobile phase as the gradient progressed and somewhat lessened the effect of the decreasing polarity and dielectric constant on the residual current. Figure 22 shows the baseline change during a blank injection and a gradient from 0 to 90% methanol with a background electrolyte concentration of 0.15% H_3PO_4 in water

Figure 22. Baseline during Gradient Program with Blank Injection

Solvent A: 0.15% H_3PO_4 in H_2O

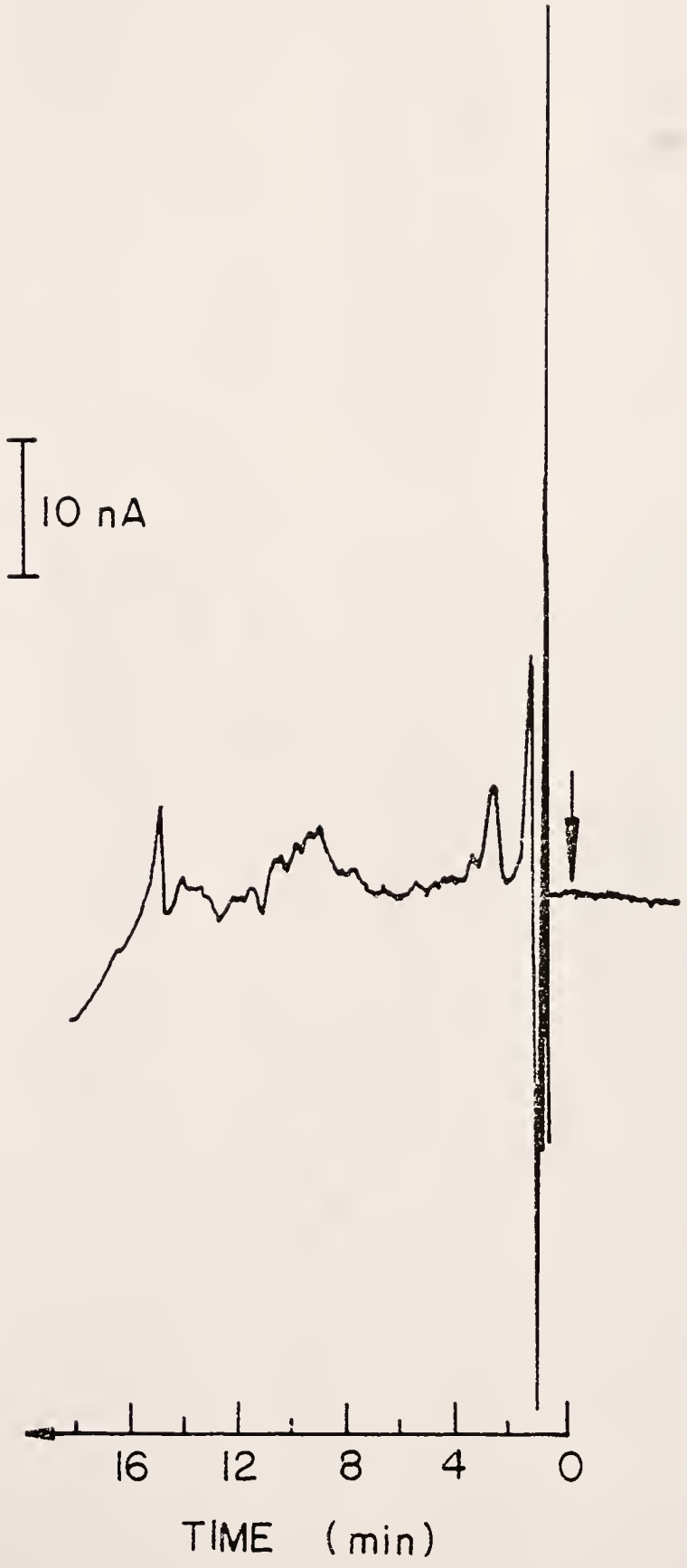
Solvent B: 0.20% H_3PO_4 in methanol

Gradient Program: initially 100% A, then immediate linear ramp to 40% B over 8 min, to 60% B over 3 min, and to 90% B over 7 min.

Flow rate: 2.0 mL/min; working electrode: glassy carbon electrode; E = 1.4 V vs. Ag/AgCl

Column: Altex Ultrasphere ODS, 150 × 4.6 mm

Injection volume: 10 µL blank (1% ammonium hydroxide in methanol)


10 nA
16
12
8
4
0
TIME (min)

and 0.20% H_3PO_4 in methanol. It should be stressed that the potential of the working electrode during this gradient program was +1.4 V and that lower working potentials should show even less baseline shift. Also, no extraordinary efforts were made to purify the water used, and some of the peaks observed are undoubtedly from trace organic compounds which had adsorbed at the top of the column. Figure 23 shows the rapid gradient elution separation of the 7 thyromimetic iodoamino acids which are shown in Figure 24. The peak at 3.5 min is from the ammoniacal methanol used to dissolve the sample. This chromatogram demonstrates the potentially powerful applications of gradient elution LC/EC.

Isocratic Separations

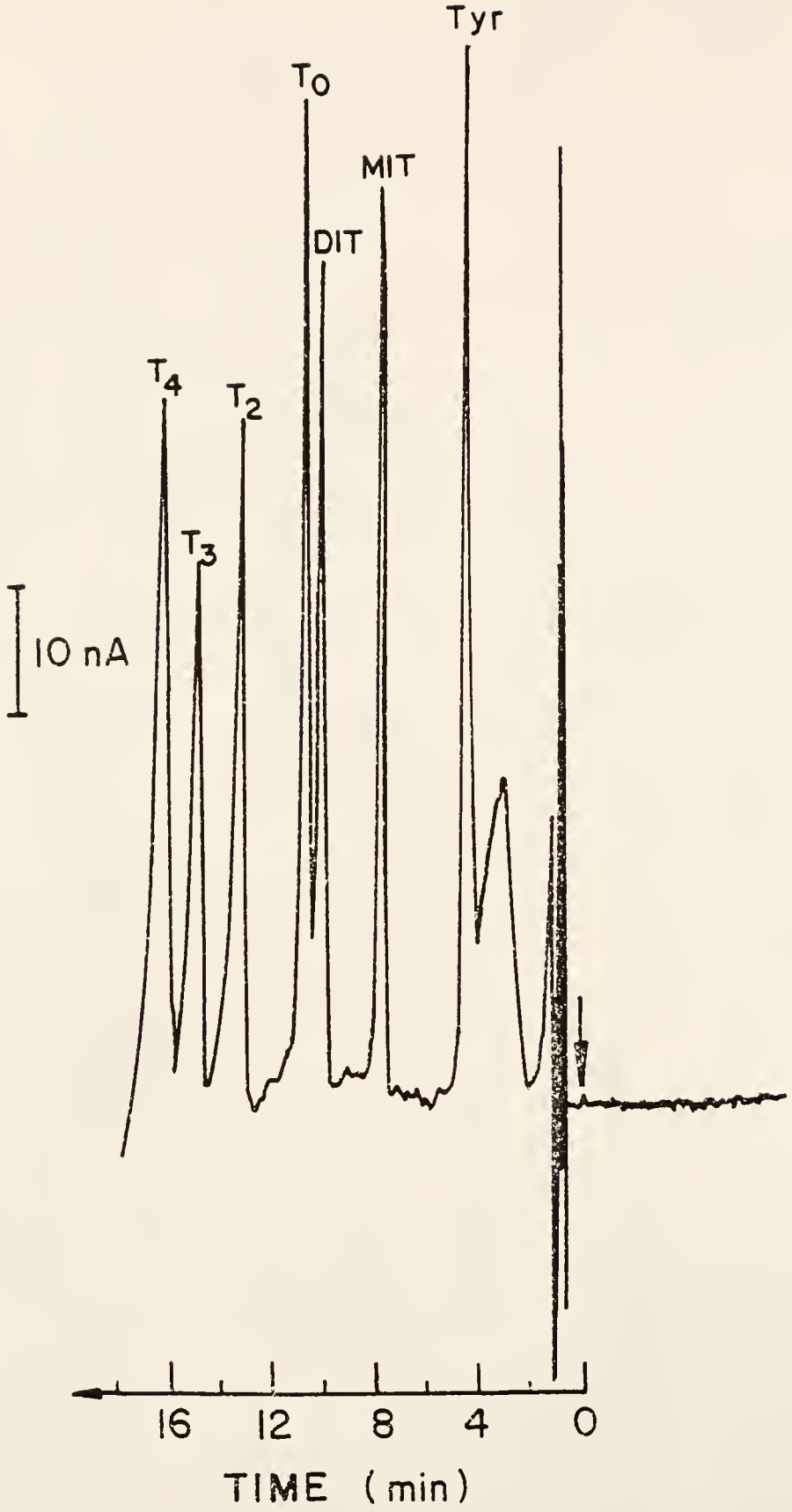
To maximize the signal-to-noise ratio of an electrochemical detector, the applied potential should be held at the minimum value at which the current reaches the limiting current plateau of the analyte ($E_{plateau}$). This potential can be quickly estimated from cyclic voltammetry [25] and can then be determined precisely from hydrodynamic voltammogram (HDV) in which the current is measured vs. applied potential point by point. A cyclic voltammogram for T_0 [81] is shown in Figure 25. Hydrodynamic voltammograms for T_2, T_3 and T_4 are shown in Figure 26. Each point is the average signal from two, 5 µL injections of a 50 ppm solution (0.25 µg/injection) at a flow rate of 1 mL/min. As seen in Figure 26, a potential of 1.2 V is a reasonable potential

Figure 23. Separation of Seven Thyromimetic Iodoamino Acids

Column: Altex Ultrasphere ODS, 150 × 4.6 mm

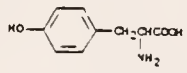
Injection: 10 µL of 20 ppm Tyr, 40 ppm MIT, 70 ppm DIT, 25 ppm T_0, 60 ppm T_2, 60 ppm T_3, 200 ppm T_4

Other conditions are as in Figure 22.

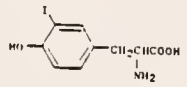

Tyr
T0
MIT
DIT
T4
T2
T3
10 nA
16
12
8
4
0
TIME (min)

THYROMIMETIC IODOAMINO ACIDS AND RELATED COMPOUNDS

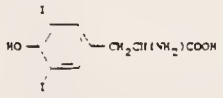
D,L-TYROSINE (TYR)



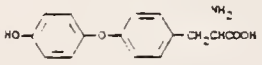
3-IODO-L-TYROSINE (MIT)



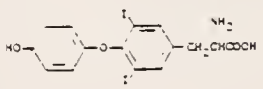
3,5-DIIODO-L-TYROSINE (DIT)



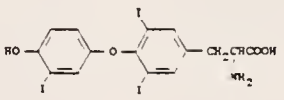
D,L-THYRONINE (T_0)



3,5-DIIODO-D,L-THYRONINE (T_2)



3,3',5-TRIIODO-L-THYRONINE (T_3)



L-THYROXINE (T_4)

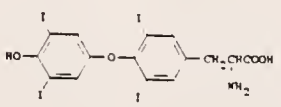


Figure 24. Thyromimetic Iodoamino Acids Used in This Study

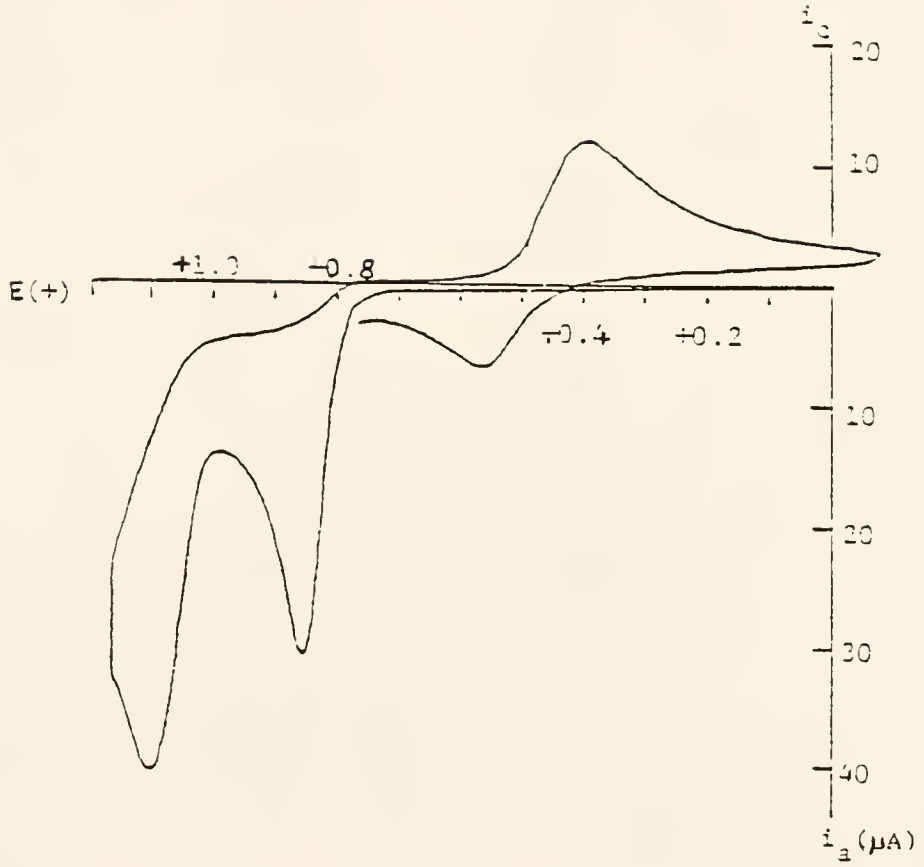


Figure 25. Cyclic Voltammogram [81]

Sample: T_0; medium: 1 M H_2SO_4; concentration: 5 mg/25 mL; scan rate: 200 mV/sec; working electrode: carbon paste electrode; reference electrode: Ag/AgCl; instrument: CV-1A

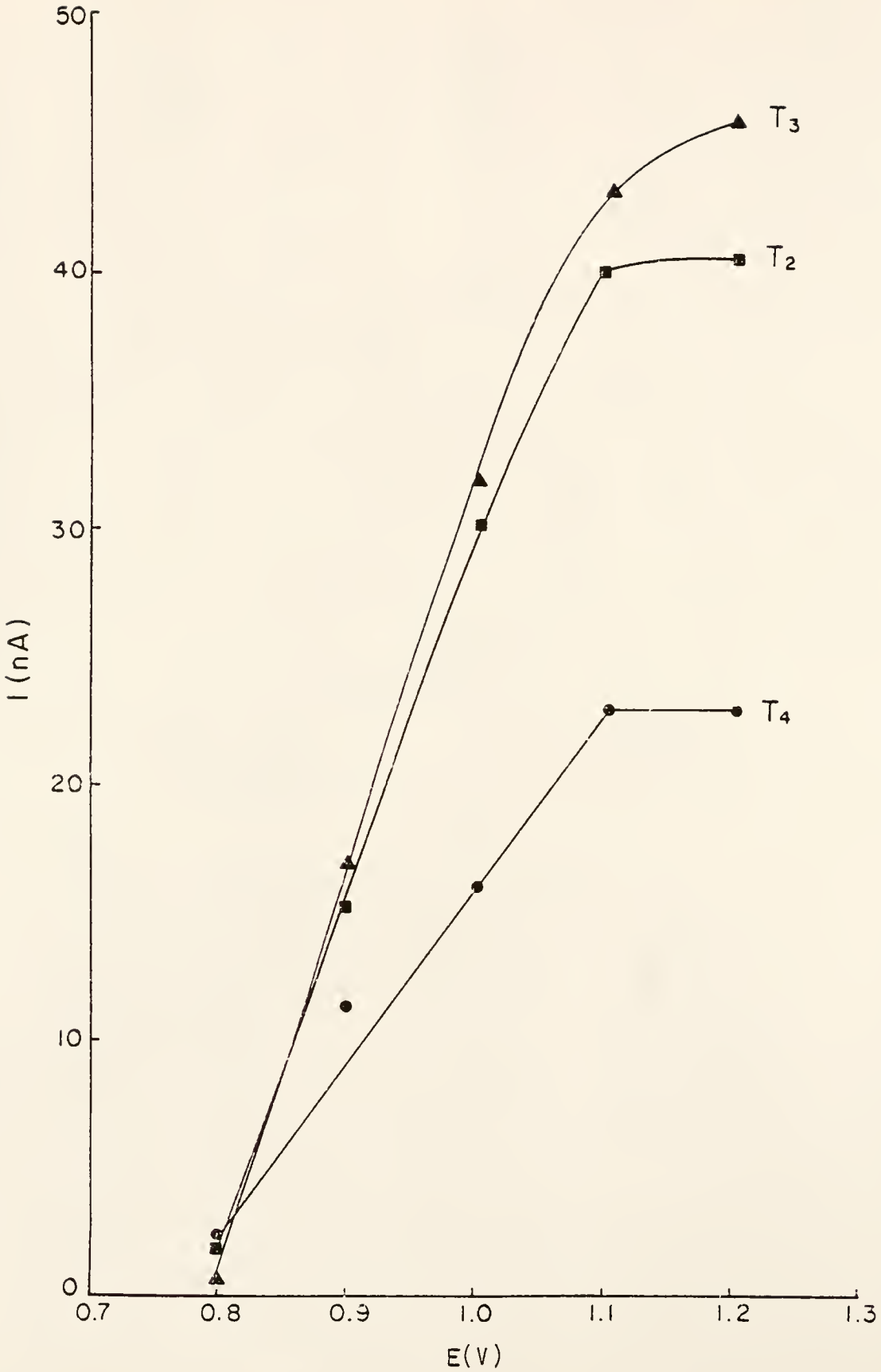
Figure 26. Hydrodynamic Voltammogram for T_2, T_3 and T_4

Mobile phase: (70:30:0.2) (CH_3OH:H_2O:H_3PO_4)

Flow rate: 1.0 mL/min

Column: Altex Ultrasphere octyl, 250 × 4.6 mm

Injection: 5 µL of 50 ppm solution


50
40
30
20
10
0
I (nA)
0.7
0.8
0.9
1.0
1.1
1.2
1.3
E(V)
T3
T2
T4

for measurement of these compounds, and the other thyromimetic iodoamino acids were also found to produce large signals at this potential.

Analytical calibration curves, current vs. concentration, for T_2, T_3 and T_4 are shown in Figure 27. Each point is the average signal of two, 5 µL injections of standard solutions with an applied potential of 1.2 V. The analytical figures of merit for T_2, T_3 and T_4 are given in Table 6. As can be seen, the limits of detection are in the subnanogram range, and the LOD's for Tyr, MIT, DIT and T_0 should be even lower, as the signal for these compounds is greater than for an equal concentration of T_2, T_3 or T_4. Figure 28 shows a rapid isocratic separation of T_0, T_2, T_3 and T_4.

Assay of T_4 Preparations

To demonstrate the usefulness of electrochemical detection for these compounds, both T_4 tablets and intravenous solutions were analyzed. For the determination of T_4, a calibration curve was prepared using standard solutions. Each standard was measured two times, and the average peak height signal of these two measurements was used for the calibration curve. The average signal of 5 measurements was used for the unknowns. The average amount of L-thyroxine per tablet was found to be 24.3 µg (25 µg/tablet claimed), and the injectable solution was found to contain 704 µg (500 µg claimed). The reason for this large excess is unknown.

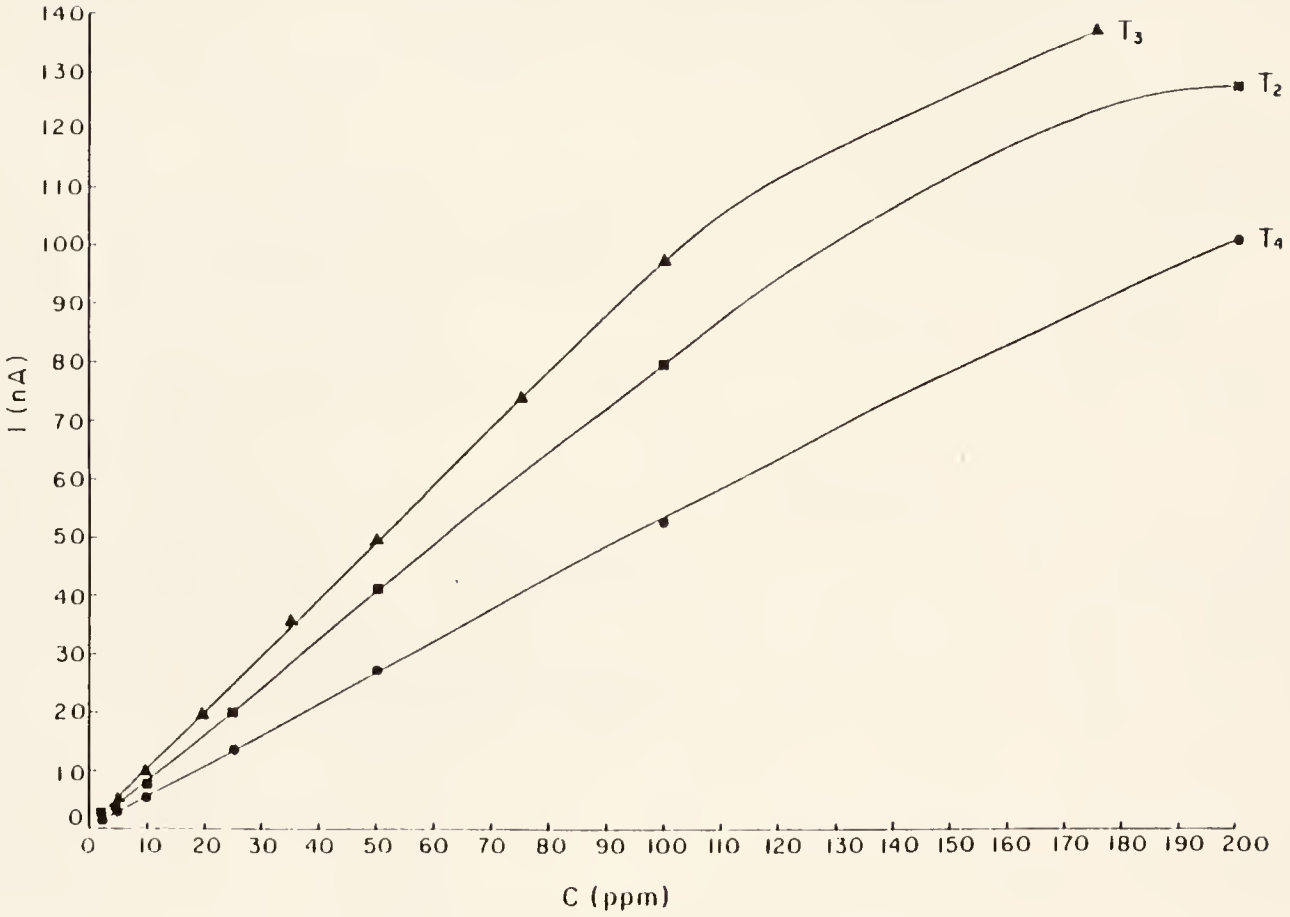


Figure 27. Analytical Curves for T_2, T_3 and T_4

E = +1.2 V

Other conditions are as in Figure 26.

Table 6. Analytical Figures of Merit for T_2, T_3 and T_4. Flow rate: 1.0 mL/min; column: Altex Ultrasphere octyl, 250 × 4.6 mm; injection: 5 µL of 50 ppm solution; E = 1.2 V vs. Ag/AgCl.

Mobile phase: (70:30:0.2)(CH_3OH:H_2O:H_3PO_4).

Compound	Limit of Detection (ppm)	Limit of Detection (ng)	Upper Limit of LDR (ppm)	Upper Limit of LDR (ng)	Sensitivity (nA/ppm)	Sensitivity (nA/ng)	Log-log Slope	t_R (min)
T_4	0.026	0.13	200	1000	0.460	0.092	0.97	9.0
T_3	0.012	0.062	100	500	0.984	0.197	0.98	7.0
T_2	0.014	0.074	100	500	0.805	0.161	1.00	6.0

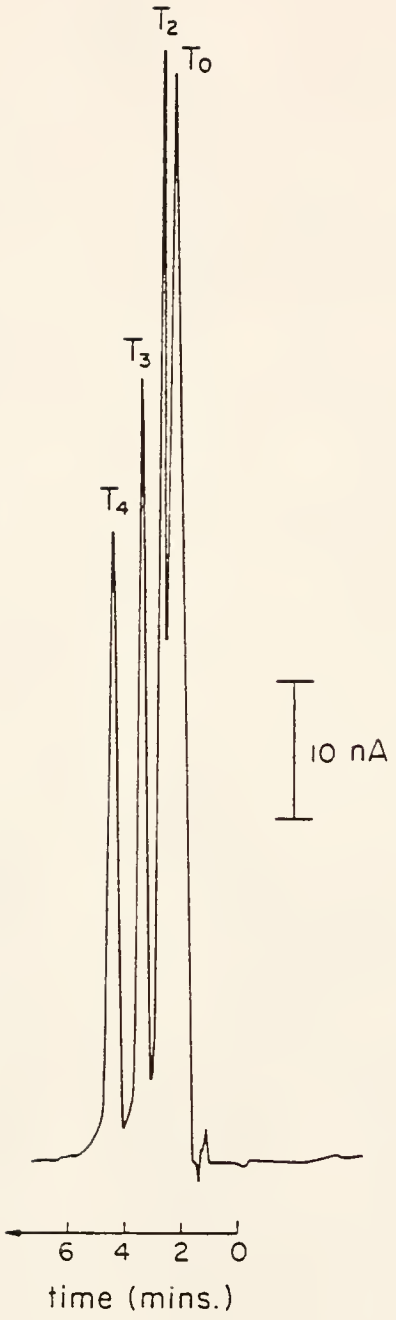


Figure 28. Isocratic Separation of T_2, T_3 and T_4

Flow rate: 2.0 mL/min; E = +1.2 V

Other conditions are as in Figure 26.

As amperometric detectors, particularly with glassy carbon working electrodes, are known to undergo changes in sensitivity with time, it is necessary to run two or more standards daily to reestablish the slope of the working curve. A study of reactivation methods for solid electrodes used in LC/EC and flow injection analysis has recently been made [33]. It is also necessary to prepare fresh standards daily, as the compounds were found to slowly decompose, with old standards showing a small peak eluting before the T_4 peak.

CHAPTER SEVEN

CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

Conclusions

A method for utilizing gradient elution techniques with electrochemical detectors in both hydroorganic and micellar mobile phases is described. This should greatly increase the usefulness of this detection method and should serve to shorten analysis time where electrochemical detection is the method of choice. A comparison of analytical figures of merit between hydroorganic and micellar mobile phases shows the possibility of greater linear dynamic ranges for electroactive components in micellar mobile phases. The unique selectivity of electrochemical detection can provide an effective method to discriminate between coeluting components in both micellar and hydroorganic mobile phases. The selective detection is shown in hydrodynamic voltammograms for anthracene and pyrene. Limits of detection in the nanogram range in addition to the selectivity of micellar mobile phase show the sensitivity of electrochemical detection and versatility of micellar mobile phases for B-6 vitamins. A very sensitive method (electrochemical detection) for thyromimetic iodoamino acids is described. This sensitive method can provide a limit of detection in the sub-nanogram to

picogram range, depending on the type of the thyromimetic iodoamino acids. Rapid isocratic and gradient elution separations of the thyromimetic iodoamino acids are also reported.

Suggestions for Future Work

Future research in LC/EC looks promising due to the versatility of the technique and the additional selectivity, sensitivity, and signal enhancement found when micellar mobile phases are used with LC/EC. Ideas for future research are given in the following paragraphs.

Firstly, the separation of chemical compounds which interact similarly with the stationary and mobile phases is a major problem in HPLC; however, the selectivity of electrochemical methods at a properly applied potential will provide the detection of one component in the presence of another, if the signal of one component approaches zero at that potential. This properly applied potential might not be the analytical potential, which may cause a decrease in detection sensitivity. One example might be the selective detection of polyaromatic hydrocarbons (PAH) with amperometric or pulse electrochemical methods, because these compounds are typically hard to separate.

Secondly, as mentioned in Chapter Five, surfactant solutions, especially cationic surfactants, can catalyze oxidative waves [45,82]. The same literature also reported an increase in the oxidation potential of water by up to 1 V

when platinum anodes were used in a surfactant solution which was also 2 M sodium hydroxide; however, the literature did not mention what occurs in the absence of 2 M sodium hydroxide in the micellar solution. The advantages of micellar solutions can provide the means for the detection of many chemical compounds with high oxidative potentials and can increase the sensitivity of compounds that produce slight shoulders or no waves in aqueous 2 M sodium hydroxide solutions. If one assumes this is true only in micellar solutions with 2 M sodium hydroxide, however, one can add sodium hydroxide after the components elute from the column to get the benefit of this powerful detection technique.

Thirdly, the indirect determination of chemicals with no detector response can be accomplished using sensitive electrochemical methods. For example, sugars, steroids, and alkyl ammonium compounds cannot be directly detected with common liquid chromatographic detectors. The addition of small amounts of an electroactive component to the mobile phase will produce a negative peak for a nonelectroactive component. The height of this negative peak will be proportional to the concentration of nonelectroactive component band passing through a detector cell. Addition of an electroactive ion-pair reagent to the mobile phase will provide a means of detection for ionic samples of the opposite charge. For example, to separate and detect alkyl ammonium compounds, an alkyl phenol sulfonate used as an ion-pair reagent can provide the separation as well as detection by RP-LC/EC.

Fourthly, a zwitterionic surfactant molecule contains both cationic and anionic sites. Addition of the zwitterionic surfactants to the mobile phase will facilitate separation of cationic and anionic compounds in a mixture. A mixture of cationic and anionic surfactants can be separated by zwitterionic surfactants in the mobile phase.

Fifthly, as mentioned in Chapter Five, the upper limit of LDR in micellar mobile phases was higher than in hydro-organic mobile phases. The reasons mentioned were either higher ionic strength in a micellar mobile phase or the nature of surfactants. To discriminate between the two reasons, one can compare the upper limit of LDR by setting the ionic strength equal in both mobile phases, or by choosing nonionic surfactants with the same concentration of background electrolyte in both mobile phases. Any observed differences in the upper limit of LDR in two mobile phases then will be due to the nature of surfactants.

Finally, surfactants can be chemically bonded to silica gel to produce anionic stationary phase. A chemically bonded anionic surfactant stationary phase can be used for the separation of cationic compounds with a cationic micellar mobile phase, while a chemically bonded cationic surfactant can be used for separation of anionic compounds with an anionic micellar mobile phase. The competition between the ionic samples and surfactants in the mobile phase for stationary phase causes the separation of ionic compounds in a mixture.

REFERENCES

1. Martin, A. J. P.; Synge, R. L. M. Biochem. J. 35, 91 (1941).

2. Karger, B. L.; Snyder, L. R.; Horvath, C. "An Introduction to Separation Science," John Wiley & Sons, New York, 1973; p. 3.

3. Martin, A. J. P.; Synge, R. L. M. Biochem. J. 35, 1558 (1941).

4. Unger, K. K.; Becker, N.; Roumeliotis, P. J. Chromatogr. 125, 115 (1976).

5. Majors, R. E.; Hopper, M. J. J. Chromatogr. Sci. 12, 767 (1974).

6. Gilpin, R. K.; Korpi, J. A.; Janicki, C. A. Anal. Chem. 46, 1314 (1974).

7. Hemetsberger, H.; Maasfeld, W.; Ricken, H. Chromatographia 10, 303 (1976).

8. Hemetsberger, H; Kellermann, M.; Ricken, H. Chromatographia 10, 726 (1977).

9. Hemetsberger, H.; Behrensmeyer, P.; Henning, J.; Ricken, H. Chromatographia 12, 71 (1979).

10. Kikta, E. J.; Grushka, E. Anal. Chem. 48, 1098 (1976).

11. Colin, H.; Guiochon, G. J. Chromatogr. 158, 183 (1978).

12. Haleem, J. I. J. Liq. Chromatogr. 4, 1917 (1981).

13. Halasz, I.; Heine, E. Adv. Chromatogr. 4, 207 (1967).

14. Green, R. B. Anal. Chem. 55, 20A (1983).

15. Drake, B. Acta Chem. Scand. 4, 554 (1950).

16. Kemula, W. Rocz. Chem. 26, 281 (1952).

17. Stulik, K.; Pacakova, V. J. Electroanal. Chem. 129, 1 (1981).

18. Pungor, E.; Toth, K.; Feher, Zs.; Nagy, G.; Varadi, M. Anal. Lett. 8, ix (1975).

19. Kissinger, P. T. Anal. Chem. 49, 447A (1977).

20. Borman, S. A. Anal. Chem. 54, 332A (1982).

21. Delley, R. Chromatographia 9, 10 (1976).

22. Foley, J. P.; Dorsey, J. G. Anal. Chem. 55, 730 (1983).

23. Kaiser, H. Spectrochm. Acta B, 33B, 551 (1978).

24. Long, G. L.; Winefordner, J. D. Anal. Chem. 55, 712A (1983).

25. Anderson, J. L.; Weisshaar, D. E.; Tallman, D. E. Anal. Chem. 53, 906 (1981).

26. Hackzell, L.; Schill, G. Chromatographia 15, 437 (1982).

27. Sachok, B.; Deming, S. N.; Bidlingmeyer, B. A. J. Liq. Chromatogr. 5, 389 (1982).

28. Bidlingmeyer, B. A. J. Chromatogr. Sci. 18, 525 (1980).

29. Armstrong, D. W.; Nome, F. Anal. Chem. 53, 1662 (1981).

30. Dorsey, J. G.; DeEchegaray, M. T.; Landy, J. S. Anal. Chem. 55, 924 (1983).

31. LC-4 Amperometric Controller, Operation/Maintenance Manual, Bio Analytical Systems, Inc., West Lafayette, Indiana.

32. Rubinson, K. A.; Gilbert, T. W.; Mark, H. B. Anal. Chem. 52, 1549 (1980).

33. Vanrooijen, H. W.; Poppe, H. Anal. Chim. Acta 130, 9 (1981).

34. Engstrom, R. C. Anal. Chem. 54, 2310 (1982).

35. Dieker, J. W.; Van der Linden, W. E.; Poppe, H. Talanta 26, 511 (1979).

36. Fleet, B; Little, C. J. J. Chromatogr. Sci. 12, 747 (1974).

37. Swartzfager, D. G. Anal. Chem. 48, 2189 (1976).

38. Fleet, B. British Patent 1531761; U.S. Patent 4059406. EDT Supplies Ltd.

39. Stastny, M; Volf, R.; Benadikova, H.; Vit, I. J. Chromatogr. Sci. 21, 18 (1983).

40. Bockris, J. O'M.; Reddy, A. K. N. "Modern Electrochemistry," Vol. 1, 385 (1970).

41. Janz, G. J.; McIntyre, J. D. E. J. Electrochm. Soc. 108, 272 (1961).

42. Armstrong, D. W.; Henry, S. J. J. Liq. Chromatogr. 3, 657 (1980).

43. Love, L. J. C.; Skrilec, M; Habarta, J. G. Anal. Chem. 52, 754 (1980).

44. Weinberger, R.; Yarmchuk, P.; Love, L. J. C. Anal. Chem. 54, 1552 (1982).

45. Franklin, T. C.; Iwunze, M. Anal. Chem. 52, 973 (1980).

46. Yildiz, A.; Kissinger, P. T.; Reilley, C. N. Anal. Chem. 40, 1018 (1968).

47. Yarmchuk, P.; Weinberger, R.; Hirsch, R. F.; Love, L. J. C. Anal. Chem. 54, 2233 (1982).

48. Knox, J. H.; Hartwick, R. A. J. Chromatogr. 204, 3 (1981).

49. Oppenheimer, J. H. Science 203, 971 (1979).

50. U. S. Pharmacopeia, 19th revision. U.S. Pharmacopial Convention, Rockville, Maryland, p. 282 (1975).

51. Ibid., p. 286.

52. Baker, S. B.; Humphrey, M. J.; Soley, M. H. J. Clin. Invest. 30, 55 (1951).

53. Man, E. B.; Kydd, D. M.; Peters, J. P. J. Clin. Invest. 30, 531 (1951).

54. Pileggi, V. J.; Lee, N. D.; Golub, O. J.; Henry, R. J. J. Clin. Endocrinol. 21, 1272 (1961).

55. Chopra, I. J.; Solomon, D. H.; Ho, R. S. J. Clin. Endocrinol. Metab. 33, 865 (1971).

56. Gharib, H.; Ryan, R. J.; Mayberry, W. E. Mayo Clin. Proc. 47, 934 (1972).

57. McDonald, L. J.; Robin, N. I.; Siegel, L. Clin. Chem. 14, 1891 (1978).

58. Siegel, L.; McDonald, L. J.; Robin, N. I. Clin. Chem. 24, 1891 (1978).

59. Hollander, C. S. Trans. Assoc. Am. Physicians 81, 76 (1968).

60. Peterson, B. A.; Hanson, R. N.; Giese, R. W.; Karger, B. L. J. Chromatogr. 126, 503 (1976).

61. Faircloth, M. A.; Williams, A. D.; Florsheim, W. H. Anal. Biochem. 12, 437 (1965).

62. Dobias, M.; Mucha, J.; Talan, P. Radiochem. Radioanal. Lett. 33, 179 (1978).

63. Cieri, U. R.; Illuminat, J. C. J. Assoc. Offic. Anal. Chem. 60, 628 (1977).

64. Kologlu, S.; Schwartz, H. L.; Carter, A. C. Endocrinol. 78, 231 (1966).

65. Lemieux, R.; Talmage, J. M. J. Pharm. Pharmacol. 18, 94 (1966).

66. Miller, A.; Horster, F. A. Z. Krebsforsch. 87, 47 (1976).

67. Heki, N.; Noto, M.; Hosojima, H.; Takahashi, S.; Murata, T. Folia Endocrinol Jap. 52, 149 (1976).

68. Karger, B. L.; Su, S. C. J. Chromatogr. Sci. 12, 678 (1974).

69. Waters Associates Applications Highlight, #19 (1976).

70. Hearn, M. T.; Hancock, W. S.; Bishop, C. A. J. Chromatogr. 157, 337 (1978).

71. Rapaka, R. S.; Knight, P. W.; Shah, V. P.; Prasad, V. K. Anal. Lett. 12, 1201 (1979).

72. Smith, D. J.; Graham, J. H. J. Assoc. Offic. Anal. Chem. 62, 816 (1979).

73. Smith, D. J.; Biesemeyer, M.; Yaciw, C. J. Chromatogr. Sci. 19, 72 (1981).

74. Hearn, M. T. W.; Hancock, W. S. J. Liq. Chromatogr. 2, 217 (1979).

75. Nachtmann, F.; Knapp, G.; Spitzy, H. J. Chromatogr. 149, 693 (1978).

76. Hepler, B. R.; Weber, S. G.; Purdy, W. C. Anal. Chim. Acta 113, 269 (1980).

77. Bongiovanni, R.; Burman, K. D.; Garis, R. K.; Boehm, T. J. Liq. Chromatogr. 4, 813 (1981).

78. Rucki, R. J. Talanta 27, 147 (1980).

79. Bollet, C.; Oliva, P.; Caude, M. J. Chromatogr. 149, 625 (1977).

80. Su, S. J.; Grego, B.; Hearn, M. T. W. J. Liq. Chromatogr. 4, 1709 (1981).

81. C. V. Notes Bio Analytical Systems Inc., Loren Place, West Lafayette, Indiana.

82. Franklin, T. C.; Sidarous, L. J. Electrochem. Soc. 124, 65 (1977).

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Mohammad Reza Hadjmohammadi was born November 27, 1947, in Iran. He attended primary and secondary schools there, graduated from the Mashad University with a B. S. degree in chemistry in 1972, served military service, and received his masters degree in chemistry in 1975 from Tehran University. He worked as an instructor at the Chemistry Department of Abureihan University from 1975 to 1977. He came to the University of Florida, Gainesville, Florida, U.S.A., in September 1977, where he has taken some English courses and is working toward the Ph.D. degree in analytical chemistry. He is a student member of the American Chemical Society and Analytical Chemistry Division.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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December 1983

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